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THE PROPERTIES OF ANTIGENIC PREPARATIONS FROM *BRUCELLA MELITENSIS*: II. SEROLOGICAL PROPERTIES OF THE ANTIGENS.

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IN the preceding paper of this series (Miles and Pirie, 1939) we decribed the preparation of *Br. melitensis* antigen in two states of aggregation. These were referred to by the symbols [PLAPS] and AP respectively; the more complex antigen, [PLAPS], could be converted into AP by a number of treatments, which led to the separation of a lipoid mixture, PL1, and a material, S, which resembles a protein in many ways. This degradation could not be reversed.

This paper is concerned with the serological behaviour of these substances, and that of the material which can be prepared by disintegrating AP with sodium dodecyl sulphate.

SEROLOGICAL PROPERTIES OF THE SSS IN VARIOUS STAGES OF AGGREGATION.

Precipitating rabbit antisera were prepared against *Br. abortus* and *melitensis* by the injection either of multiple doses of dead, agar-grown organisms (a total of 40×10^9 in each course of injections), or of single doses (10^8) of living broth cultures. The prolonged immunization with dead bacteria often required for the production of precipitins, allowed ample time for the production of antibodies to minor antigenic components. In most animals precipitins for homologous and heterologous SSS appeared together, though the heterologous precipitins were the more easily inactivated by dilution of the sera. Some bleedings, however, were active only with extracts from the homologous bacteria, and it is possible that the failure of some of Higginbotham and Heathman's (1936) *Brucella* fractions to precipitate with heterologous antisera was due to the use of such apparently monospecific sera. In view of the prevalence of agar-derivatives in bacterial fractions, the exclusion of possible agar precipitin effects is important; it was found that the serological behaviour

of antisera prepared against agar- and broth-grown organisms was qualitatively identical, and there is no reason to suppose that agar-precipitins complicated the serological picture.

Fractionation of the bacteria was checked throughout by precipitin tests of three-fold dilution series, usually 1 : 1000 to 1 : 2,187,000, against an equal volume of constant-antiserum dilution. At 56° C., with antiserum 1 : 10 to 1 : 25, precipitation was marked in 1 hour, and the end-point maximum at 4 hours. The optimal (most rapidly particulating) concentration of antigen was determined by readings every 5–10 minutes. No attempt was made to increase the end-points by spinning out invisible precipitates to produce a visible disc. Our best preparations gave a visible, settled precipitate at 1 : 2·5 million. Higher titres were found in many titration series, but were due to cumulative dilution errors, since accurate dilution of SSS in litre volumetric flasks reduced the apparent activity to the 1 : 2·5 million limit.

Unless otherwise stated, end-points and optimal concentrations of antigen are recorded throughout as the dilution of antigen reacting with an equal volume of serum. Final precipitating concentrations are half these amounts, *i.e.* the final titre of purified AP is 1 : 5 million. The optimally flocculating concentration of antigen bore a constant ratio to the concentration of serum, but the antigen end-point was approximately constant with serum dilutions from 1 : 5 to 1 : 50, *i.e.* it was independent of the antiserum concentration in this range. Hence, with a given specimen of serum, end-points could be used as a measure of activity and a guide in chemical fractionation (*cf.* Table IV). The relationship of various agglutinating and precipitating antigen-antibody systems is shown in Table I, which summarizes experiments on smooth antisera from 15 rabbits.

The Serology of AP.

The AP precipitation zone has a 700-fold range, from about 1 : 3000, with an optimum at about 1 : 27,000, and an E.P. of 1 : 2,187,000 (Table I, col. 1). The optimum is equivalent if absorptions are carried out at 56° C. ; the greatest bulk of precipitate after 4 hours occurs with antigen excess. The combination at 37° C. is slow and incomplete after 12 hours ; combination at 0°–2° C. is incomplete after 4 days. At 56° C., AP precipitates from antigen concentrations in excess of equivalence were maximum in 4 hours, and to a certain extent redissolved after 24 hours (col. 2) ; the precipitates of some preparations appeared as a coarse fibrous net festooned across the lumen of the tube, suggesting an orientation within the precipitate.

We hoped to separate the wide zone of AP precipitation into two zones, due to the hypothetical A and M antigens of Wilson and Miles (1932), by precipitin-absorption of antisera active with both *melitensis* and *abortus* SSS. Most of these sera reacted strongly at 1 : 10, but since in this dilution minor precipitins might be inactive, the sera were absorbed neat or diluted at most 2-fold, in order that any residual antibodies might be detectable by the precipitin reaction. The sera were absorbed at the constant-antibody optimum. With dilute sera this was equivalent, the antigen-antibody ratio was constant, and an excess of either antigen or antibody could be detected in the supernatant

TABLE I.—*Typical Constant-antiserum Titrations (at 56° C.) of Bacterial Fractions with* Br. melitensis *Serum.*

	1.	2.	3.	4.	5.	6.*	7.*
Antiserum absorbed by	0	0	0	*Br. abortus SSS*	0	0	0
Antigen	AP	AP	*Br. abortus SSS*	AP	[PLAPS]	AP	[PLAPS]
Time (hours)	4	24	4	4	4 and 24	4	4 and 24
Antigen concentrations.							
1 : 110	−	−	−	−	+++	−	++
1 : 330	−	−	−	−	++++	−	+++
1 : 1000	−	−	±	−	++++	−	op. ++; tr.
1 : 3000	±	−	±	−	+++	op.; tr.	op. +++; −
1 : 9000	+++	−	+++	±	+++	op. +; ±	op. ++; tr.
1 : 27,000	++++	+	++	++	+++	op.; +++	++
1 : 81,000	+++	+++	+	+++	++	+++	+±
1 : 243,000	+++	+++	±	+++	+	+++	+
1 : 729,000	++	++	−	++	−	++	−
1 : 2,187,000	±	+	−	±	−	±	−
1 : 6,561,000	−	−	−	−	−	−	−

The antigen concentrations are initial; final concentrations are half the recorded amount. op. = opalescent; tr. = trace; ± to ++++ = intensity of opalescence or bulk of precipitate. * Columns 6 and 7 typify the reactions with a double-zoning serum.

fluid from mixtures with either more or less antigen than this equivalent amount. With concentrated antisera, however, the optimum and the antigen- and antibody-excess mixtures were irregularly disposed and the optimal ratio no longer held. Forcible " squirt-mixing " of the antigen solution into concentrated antibody reduced these irregularities, and brought the optimal ratio nearer to its expected value. Apparently the immediate interface reactions between casually mixed antibody and antigen solutions produced heterogeneous precipitates, too compact to allow a subsequent homogeneous redistribution of antigen and antibody. In dilute systems the slower reaction and looser floccules permit re-solution and redistribution (as the re-solution of antigen-excess AP precipitates in ordinary titrations testifies) and the optimal ratio holds. For precipitin absorption a mixing device was adapted from Millikan (1936). Two 10 c.c. Record syringes of equal internal diameter are filled with antigen and antibody solutions and mounted with a common arm to operate their plungers. A 3-way tap of about 0·35 c.c. capacity is filled with saline and fitted to their nozzles. The mixed fluids are collected at the common tap after the first 0·6 c.c. has been discarded. Homogeneous mixtures are produced; and the optimally particulating zone is equivalent and the optimal ratio equals that determined in weaker antiserum titrations. Without such a device, residual precipitins may be found in the supernatant fluids from strong mixtures that contained equivalent amounts of antigen and antibody, and their presence may be fallaciously interpreted as indicating a second type of antibody. Most absorption tests were repeated two to three times; sera from several rabbits were used.

Absorption with AP.

Absorption of *melitensis* and *abortus* sera at the equivalence point of all the fractions from homologous organisms removed all homologous precipitins within a 1 : 1000 to 1 : 2,187,000 range of antigen titration. Some of the weaker fractions, notably those containing " nucleoprotein ", or protein-like residues, reacted in concentrated solution (1 : 100 to 1 : 500) with fully absorbed sera. Either a small proportion of the fraction consisted of a minor antigen, reacting optimally or near-optimally with a low concentration of a residual and distinct precipitin, or a low concentration of main antibody was present, insufficient to produce visible precipitates with highly dispersed solutions of antigen, but capable of "agglutinating" particles of substances like nucleo-proteins contaminated with the main antigen. The second explanation is more likely, since all active " nucleo-protein " fractions on analysis yielded SSS that was similar, serologically and chemically, to AP. The precipitin reaction is the least sensitive of the available serological tests for residual *Brucella* antibodies, and attempts to sensitize soluble antigens to presumed sub-precipitating doses of antibody were unsuccessful. Either minor antibodies were not present as precipitins in the absorbed homologous sera, or the chemical fractionation did not separate A from M.

The optimum and end-point concentrations of heterologous AP were higher than those of homologous AP with a given serum (Table I, col. 3); absorption by heterologous antigens at their equivalence point had little effect on the

homologous end-point, but widened the inhibition zone and shifted the optimum into regions where the antigen was 3–6 times as dilute (*e.g.* Table I, col. 4), *i.e.* precipitins for antigens present only in concentrated homologous antigen solutions, had been removed. The result of absorbing monospecific agglutinating sera by smooth, partially rough and partially extracted bacteria suggests that the minor antigen of *Br. melitensis* is about 5 per cent. of the total smooth antigen present (Miles, 1939). It is therefore to be expected that the strongest and most abundant precipitins would be for the major antigen ; and a *melitensis* serum absorbed by *abortus* SSS should precipitate relatively weak solutions of the *melitensis* SSS monospecifically. This was so, for the *melitensis* sera were cleared of *abortus* precipitins by absorption with *abortus* SSS at its optimum. Since neither serum nor antigen was rigidly definable in terms of the A or the M antigens, the absorbed sera had no more than an apparent *melitensis* monospecificity. Moreover, the precipitation of a given concentration of *melitensis* AP by these residual antibodies could be inhibited by *abortus* SSS 30–50 times as concentrated and, unless we assume that *abortus* SSS contains M as well as A antigen (*i.e.* the hypothesis we are testing by chemical fractionation), there is no valid evidence that the absorbed sera consisted of anti-M precipitins alone. The M-quality of *melitensis* AP could not, therefore, be safely measured by titrations with absorbed precipitin sera. An indirect measure was employed, depending on the monospecificity of agglutinating sera. Monospecific agglutinating sera, prepared by partial absorption with heterologous suspensions, contain in the range of monospecific concentrations only 8–16 doses of antibody capable of agglutinating an equal volume of 10^9/c.c. suspension (see Miles, 1939). Suitable dilutions of bacterial fractions in 0·2 c.c. were mixed with two doses of monospecific agglutinin in 0·2 c.c., held for 1 hour at 56° C., and 0·4 c.c. bacterial suspension was then added in the usual way. The inhibiting power of a fraction is expressed as the minimum concentration inhibiting two such doses.

TABLE II.—*Inhibition of Monospecific Agglutination by Fractions from* Brucella.

Fraction.	Inhibiting titre for monospecific agglutinins.		Ratio of inhibiting titres.
	Br. melitensis.	*Br. abortus.*	
Br. abortus SSS . . .	1 : 100–1 : 200 .	1 : 5000 .	25 or 50 : 1
Br. melitensis AP . .	1 : 16,000 .	1 : 200 .	1 : 80
,, [PLAPS] .	1 : 5000 .	.. .	..

The inhibition titres in Table II indicate that AP is three times as effective as [PLAPS] in inhibiting *melitensis* agglutination. The ratio of homologous to heterologous inhibiting titre is of the same order : 80 for *melitensis* AP and 25–50 for *abortus* SSS. If these represent the ratios of A to M in the SSS for the two species, the *melitensis* A : M ratio is substantially greater than the 1 : 20 found by agglutination tests. The *abortus* A : M ratio is smaller, probably because it was not a purified fraction, but contained its full quota of M antigen. Compared with [PLAPS], the greater titre of *melitensis* AP may be due to the removal of some A antigen during repeated fractionation.

The results of these tests remove any ambiguity in the serological distinction between *melitensis* and *abortus* SSS, and indicate that each SSS, like the surface of the bacteria from which it is derived, contains a mixture of A and M antigens. The small amount of the minor component in each SSS makes it likely that strong residual precipitins in sera absorbed with heterologous SSS are to a large extent monospecific.

The Serology of [PLAPS].

The precipitating zone of freshly prepared [PLAPS] lies between 1 : 55 and 1 : 243,000 (Table I, col. 5). Many crude bacterial fractions gave this broad zone and a relatively low end-point and presumably contained [PLAPS]. The precipitates in antigen excess, unlike those of AP, do not redissolve with prolonged heating at 56° C. The treatment of [PLAPS] with dodecyl sulphate will be described below. It leads to a great diminution in particle size, but there is apparently no loss of chemical constituents. The resulting material of relatively low molecular weight will be referred to by the symbol PLAPS. The degradation of [PLAPS] to PLAPS, or of [PLAPS] to AP is accompanied serologically by an increasing end-point, an extension of the inhibition zone into the 1 : 3000 region, and the solubility of antigen-excess precipitates in 24 hours at 56° C. (Table I, *cf.* cols. 5 and 1 ; Table III).

The coarse, fibrous, net-like precipitate occasionally seen in antigen-excess precipitates of AP is common in antigen-excess precipitates of [PLAPS] ; but with a few antisera, the fibrous precipitate occurred only in high concentrations of [PLAPS] (1 : 100 to 1 : 1000) and the broad [PLAPS] precipitation zone became double, separated by a zone of intense opalescence that persisted for two days at 56° C. without proceeding to macroscopic precipitation (Table I, col. 7). With such "double-zoning" sera, AP gave very little opalescence and a low optimum (col. 6). At the lower of the two [PLAPS] optima, all the antigen is precipitated, and the remaining antibody produces a non-precipitating opalescence with [PLAPS], but does not react with AP. At the upper optimum, about 1 : 200 to 1 : 1000, all precipitins are removed, and the supernatant fluid contains antigen which precipitates like AP to one-twentieth the titre of the original [PLAPS]. [PLAPS] which has been frozen and thawed, or left standing in solution for some time, gives upper optima of decreasing intensity, and the lower optimum increases in intensity, with an increasing end-point, suggesting a gradual breakdown of [PLAPS] into a mixture of [PLAPS], PLAPS and AP. As we have stated in the previous paper, a part of this mixture sometimes recombines in a different manner to form complexes sufficiently large to be sedimented by centrifugation at 3000 r.p.m. for many hours, and these large aggregates may also complicate the nature of the precipitation zone.

The upper optimum is sharper and a large bulk of precipitate may form rapidly ; it occurs in antigen 240 times as concentrated as that in the lower optimum. The precipitate usually consists of fine granular particles that settle slowly. During the late stages of fine particulation a coarse obvious shimmer appears, unlike the faintly visible shimmer exhibited by the antigen solution alone. Magnified 800 times under dark ground illumination a 1 mg.

per c.c. solution of sterile [PLAPS] consists of a milky suspension of shimmering, ill-defined particles that give the impression of having greater length than breadth. Among these a few faintly outlined rods and plates can be distinguished ; their length is apparently about 0·1–0·3μ, but there is no real image. On the addition of antiserum the particles immediately become opaque and appear as bright dots. The larger particles appear as approximately square plates ; some are joined in pairs along one edge to form a larger plate bent at an angle of about 140°. The particles and plates then clump into more or less elongated masses from 2–5 μ wide and 5–15μ long. These masses vary in shape with the preparation of [PLAPS], and may be thin rods and spindles or stumpier rods of 2–6μ. At this stage the shimmer is apparent to the naked eye. Further clumping of these rods is usually indiscriminate, producing a suspension of naked-eye particles which show no shimmer on agitation. The intense opalescence in the intermediate zone (Table I, col. 7, antigen 1 : 3000) is produced by particles and " plates " that never clump in groups of more than two or three. It may be mentioned here that the change from [PLAPS] to PLAPS or to AP is accompanied microscopically by an almost complete disappearance of the milky suspension of ill-defined particles, and only a few opaque masses appear on the addition of antiserum. There is also loss of anisotropy of flow and of the power to form these orientated antigen-antibody complexes. These facts suggest that the formation of the orientated precipitates with specific antibody is dependent on the integrity of large particles of antigen which have a definite polarity. The underlying theory of anisotropy of flow is still uncertain, but it can safely be assumed that fluids exhibiting the phenomenon generally contain particles that are birefringent and that depart markedly from the spherical shape, *e. g.* which are rods or plates. The upper [PLAPS] zone apparently partakes more of the nature of an "agglutination" of relatively large particles. Like agglutination of bacteria, it is produced by antisera (such as result from a single intradermal dose of AP) that are too weak to precipitate solutions of AP.

In the light of this hypothesis of aggregated and disaggregated antigen and of the result of absorption tests at the two optima, the double-zoning may arise from a mixture of two monodisperse systems of the same antigen. This seems unlikely for the following reasons: Each system, titrated singly, would precipitate optimally in a concentration dependent on the antigenic surface per unit weight, and the more dispersed antigen would be optimum in a lower concentration by weight than the less disperse antigen. In the titration series of a mixture of the two, however, the antibody would combine with both types of particle, and each type would interfere with the optimal sensitization of the other. The result would be a merging of the optima ; for at the expected lower optimum the utilization of part of the available antibody by the less disperse antigen (and consequently the resultant shift of the optimum into less concentrated solutions) is small, whereas at the high optimum the utilization by the more disperse antigen of part of the antibody, available for the optimum sensitization of the less disperse antigen, would be relatively very great (since the surface per unit weight is greater). Consequently the upper optimum is shifted until it is close to the lower. Moreover, since in these antigenic solutions all degrees of dispersion are more likely than a cluster of

size-frequences into two relatively sharp modes, a single broad zone of precipitation is to be expected from such a system.

Alternatively, [PLAPS] may differ qualitatively from AP, and react with one antibody in a different way ; or the two zones may represent two separate antigen-antibody systems. The evidence presented below favours the first of this last pair of alternative views.

Absorption with [PLAPS].

Table III typifies the result of several experiments absorbing a *melitensis* precipitating serum with varied concentrations of *melitensis* and *abortus* fractions. The converse experiments with an *abortus* serum give essentially similar results. The residual antibody was tested against homologous and heterologous AP, and against smooth *melitensis* and *abortus* suspensions.

At the equivalence point of an AP + *melitensis* serum system (Tubes 4 and 5) both homologous and heterologous precipitins are absent. The smooth agglutinins are reduced 10-fold. At the point of maximum precipitation (Tube 3) there is excess of AP, but the agglutinins are at a minimum with *abortus* agglutinins in excess. With stronger absorbing doses the agglutinating titre of the supernatant rises, presumably owing to the presence of soluble antigen-antibody complexes that alter the stability of the bacterial suspension. Absorption of an *abortus* serum by AP shows a higher equivalence point (Tubes 9 and 10) at which *abortus* precipitins are present and *abortus* agglutinins in excess. In Tube 8, at maximum precipitation, the *melitensis* agglutinin titre rises again, but the *abortus* is still decreasing. The residual *melitensis* agglutinins in Tubes 3 and 9 can be inhibited by excess of AP ; 1 mg. will inhibit 5000 agglutinin doses of unabsorbed serum but only 12 doses of fully absorbed serum. Apparently the smooth AP contains very little antigen corresponding to these residual agglutinins. On the other hand, concentrated [PLAPS] removes all antibodies for *abortus* and *melitensis* bacteria (Tube 14), though more dilute solutions leave, in *abortus* sera, an excess of *abortus* precipitins and agglutinins (as AP does in Tube 9). Similar absorption tests with other specimens of [PLAPS] gave more complex and less readily interpretable results. [PLAPS] cannot be serologically defined without a more exact definition of the rough antigens. Most preparations of [PLAPS] absorb or inhibit the action of the residual agglutinins left after smooth or rough *Brucella* antisera have been cleared of AP antibodies. The antigen responsible for these residual reactions is present in large amounts on the surface of semi-rough or rough organisms. It stimulates no precipitins, for extracts from *Br. paramelitensis* are not precipitated by the strongest smooth or rough antisera, even those prepared by prolonged immunization with massive doses. (Prolonged immunization with R organisms yields precipitins, but these are late responses to traces of smooth AP, and are completely removed by AP absorption.)

[PLAPS] behaves as though it contained the antigenic fragments of the whole bacterium. [PLAPS] from *Br. melitensis* inhibits the agglutination both of SR *abortus* and of SR *melitensis* to the same degree. Tube 9 (Table III) shows that the monospecificity of agglutination and that of precipitation are

TABLE III.—*Examples of Graded Absorption of Precipitating Antisera by Fractions from* Brucella.

Tube.	Absorbing dose.	Two-hour precipitate.	Supernatant fluids tested against—					
			Br. melitensis serum.	*Br. melitensis* AP.	*Br. abortus* serum.	*Br. abortus* SSS.	*Br. melitensis* suspension.	*Br. abortus* suspension.
1	1 : 200	+	++++	−	+	−	Not done.	
2	1 : 1000	+++	++	−	+	−	500	320
3	1 : 5000	+++++	+	−	−	−	15	60
4	1 : 25,000	+++	±	−	−	−	500	450
5	1 : 125,000	+++	−	+±	−	+	2500	2000
6	1 : 625,000	+±	−	++	−	++	6400	4000
7	*nil*	−	−	++	−	++	4800	2500
8	1 : 1000	+++++	+	−	±	−	480	640
9	1 : 5000	+++	±	−	−	+	120	1000
10	1 : 25,000	++	−	+	−	+	3000	8000
11	1 : 125,000	±	−	+	−	+	6000	5120
12	1 : 625,000	tr.	−	+	−	+	5120	6000
13	*nil*	−	−	+	−	+	7000	9000
14	1 : 1000	++++	+++	−	+	−	<5	<5
15	1 : 5000	++++	±	−	−	−	60	40
16	1 : 25,000	+++	−	−	−	−	640	450
17	1 : 125,000	++	−	+	−	−	3000	1920
18	1 : 625,000	tr.	−	++	−	−	2500	2000
19	*nil*	−	−	++	−	−	4000	2000

The supernatant fluids were tested against antigen and antibody solutions and against bacterial suspensions of Y6 and K25. Tubes 1–7 *Br. melitensis* serum absorbed by *Br. melitensis* AP. Tubes 8–13 *Br. abortus* serum absorbed by *Br. melitensis* AP. Tubes 14–19 *Br. melitensis* serum absorbed by *Br. melitensis* [PLAPS]. tr. = trace; ± to ++++++ = bulk of precipitate.

associated, and both disappear with a larger absorbing dose ; this is additional evidence that the monospecificity is associated with AP, and not with the antigens responsible for the residual agglutination. This residual agglutination is apparently due to the R antigen and antibody, for in absorbed sera with maximum reduction of smooth agglutinins it was found that the titre with S suspension is 1/32 of the unabsorbed S titre, but the R titre is only 1/8 of the unabsorbed R titre.

The specificity of the residual antigen may depend on a separate substance which, since it never stimulates precipitins, is not serologically recognizable when liberated during the disaggregation of [PLAPS] ; alternatively, it may depend upon a peculiarity in the spatial arrangement of the smaller antigenic units from which [PLAPS] is built. The anisotropy of flow, both of [PLAPS] and [PLAPS]-antibody complexes, offers some support for this second speculation. In this case, however, the [PLAPS] antibody should contain the anti-AP groupings and therefore be completely removable by AP-absorption. Inferences must be drawn with caution from results of [PLAPS] absorption, for the native antigen is unstable, and even the heating of the absorption mixture may lead to the liberation of AP, with a consequent upsetting of all the quantitative relationships upon which the qualitative characteristics of [PLAPS] are based. The problem is still under investigation.

Serological Purification.

We have attempted both to purify and to fractionate AP by specific serological precipitation from dilute solution. Optimal doses of fairly pure AP were precipitated at 56° C. and the precipitate digested with trypsin and the AP recovered by $(NH_4)_2\ SO_4$ precipitation. Ten c.c. of serum in 100 c.c. saline were mixed with 23 mg. of AP in 100 c.c. saline, and held successively at 56° C. for 70 min. and at 0° C. overnight. The digestion of the gently-washed precipitate with 25 mg. pancreatin for 4 days at 37° C. yielded 10 mg. AP. The optimum increased from 1 : 18,000 to 1 : 54,000 and the E.P. from 1 : 480,000 to 1 : 1,460,000—a value near the maximum E.P. attainable. This experiment was repeated by precipitating two equal volumes of serum with the same volumes of 1·4 per cent. and 14 per cent. AP solutions respectively. These represented equivalent and 10 times equivalent amounts of AP. At the same time 30 mg. of AP were digested with trypsin, without treatment with serum. Table IV shows the results. The AP is expressed in cols. 4 and 5 as the reciprocal of the precipitin end-point multiplied by the weight in milligrams. The tryptic digestion alone was as effective as the serum precipitation in removing impurities. The fraction recovered from the zone of antigen excess did not differ materially from the optimal fraction. The end-points were the same, and both reacted alike with *abortus* sera. With *melitensis* sera, the precipitation zone of the optimal fraction was the narrower ; but the broader spread of precipitation of the 10 times optimal fraction was not separable into two zones, and there was no other evidence of even a partial serological fractionation into A and M antigens.

TABLE IV.—*A Comparison of* Br. melitensis *SSS prepared by Serological Fractionation and by Tryptic Digestion.*

Method of purification.	Weight in mg.		Total SSS (Weight × 1/E.P.)	
	Used.	Recovered.	Used.	Recovered.
Serum precipitation of equivalent concentration	14	6·8	8,400	6,500
Serum precipitation of 10 × equivalent concentration	140	35·0	84,000	35,000
Tryptic digestion	35	18·0	18,000	18,000

THE DISAGGREGATION OF [PLAPS] AND AP BY SODIUM DODECYL SULPHATE.

An account of the disintegration of tobacco mosaic virus by incubation with sodium dodecyl sulphate has already been published (Sreenivasaya and Pirie, 1938), and it has been shown that disintegration leads to a separation of the protein from the nucleic acid and to a complete loss of the ability to precipitate with immune sera. Both [PLAPS] and AP are very easily disaggregated by similar treatment, but in these cases, although the disaggregated material can no longer be sedimented by centrifuging at 16,000 r.p.m., its precipitation with specific antisera is unaltered. On the system of symbols used in this paper disaggregated [PLAPS] is called PLAPS, it is not necessary to use a symbol for disaggregated AP.

Veronal buffer has been used in these experiments as in those of Sreenivasaya and Pirie (1938), although it is not strictly necessary, since the statement made in that paper that dodecyl sulphate precipitates with phosphates, etc., is incorrect. When a solution is made up containing 1·3 per cent. of [PLAPS] and 0·5 per cent. of dodecyl sulphate at pH 8·2, there is an immediate disappearance of the opalescence that is characteristic of the native antigen solution. If, however, the dodecyl sulphate is used at only half this concentration, the opalescence is stable for some hours at room temperature. The course of the action can therefore be followed centrifugally on samples of a mixture containing 0·5 per cent. of dodecyl sulphate, if these are diluted with two volumes of water, and it can be followed serologically at greater dilutions. When samples are centrifuged, after various periods of incubation, it is found that the amount sedimentable in 1 hour at 14,000 r.p.m. falls off rapidly, and becomes negligible after incubation for half an hour at 37° C. At this point the precipitate with antiserum has lost completely the characteristic appearance of [PLAPS] precipitates (Table I, col. 7), and the phenomenon of inhibition by excess of antigen is shown to the same extent as by AP. This effect is illustrated in Table V.

The material that has been disaggregated in this way precipitates with its antiserum, and with ammonium sulphate, in a manner indistinguishable from that of the product made by the action of acetic acid and alcohol; it differs from that product, however, in that it still contains the lipoid mixture PL1. There is no evidence regarding the manner of linkage of this lipoid mixture

TABLE V.—*Effect of Sodium Dodecyl Sulphate on the Sedimentation and Serological Precipitability of a 1·25 per cent. Solution of [PLAPS].*

Time of incubation.	Dry weight of pellet.	Initial dilution of antigen, 1 part in—						
		3000.	9000.	27,000.	81,000.	243,000.	729,000.	2,187,000.
4 hours (1)	8·0 mg.	+++	+++	++++	++±	+±	±	−
0 ,, (2)	4·5 ,,	++	+++	+++	++±	++	+	tr.
0 ,,	1·7 ,,	tr.	+±	++±	++±	+±	±	tr.
15 min.	1·3 ,,	tr.	+	+++	++	+±	+	tr.
30 ,,	0·5 ,,	tr.	±	++±	++	+±	+	+
4 hours	0·8 ,,	tr.	±	++±	++	+±	+	±

(1) Incubated without the addition of dodecyl sulphate.
(2) Antigen diluted before the addition of dodecyl sulphate.

to the rest of the SSS, but PL1 cannot be removed by repeated precipitation of the PLAPS with ammonium sulphate, nor can it be extracted from an acidified aqueous solution with ether. In this respect it resembles the material prepared by the action of acetic acid followed by cautious distillation *in vacuo*, for there also the PL1 cannot be removed from the dissolved SSS by extraction with ether, but only by treatment with a large volume of alcohol and ether.

The disaggregation of AP has been followed in some detail, for it is not complicated by the presence of PL1 and S, and it can be reversed by the treatments used to precipitate AP. Disaggregation and its reversion have been followed by measurements of the sedimentability of the material in a centrifuge; it is also clearly recognizable from the greater opacity of aggregated or re-aggregated solutions and from the greater turgor of dialysis sacs containing a solution of disaggregated material, when compared with sacs containing aggregated material of equal concentration. There is, however, little difference between the viscosities or serological behaviour of solutions of AP in the two states.

These preparations have been studied on the ultracentrifuge by Mr. J. StL. Philpot. After disaggregation, the solution appears to be homogeneous with a sedimentation constant of $12{\cdot}3 \times 10^{-13}$ at 20° C. Pyknometric measurements show that the specific gravity of AP in either state is about 1·5; the sedimentation constant therefore corresponds to a molecular weight of 100,000–200,000 if the usual assumptions are made (*cf.* McFarlane and Kekwick, 1938). As might be expected, aggregated or reaggregated material has a higher sedimentation constant; it is, however, highly poly-disperse. The state of aggregation can be followed as before by weighing the pellet got after 3 hours' centrifuging at 14,000 r.p.m. on the angle centrifuge. In a typical experiment four 3 c.c. lots of 1 per cent. AP were centrifuged together, after thorough dialysis, with the following result:

(*a*) Starting material	17·5 mg. pellet.
(*b*) After disaggregation with dodecyl sulphate	0·5 mg. ,,
(*c*) Solution (*b*) precipitated with alcohol and acetic acid	6·0 mg. ,,
(*d*) Solution (*c*) precipitated with ammonium sulphate	17·0 mg. ,,

Disaggregated material still contains all the constituents that have been recognized in the analysis of AP ; it contains 0·4 per cent. of sulphur instead of the 0·1–0·2 per cent. that is characteristic of the best AP preparations. This may be due to bound dodecyl sulphate, but there is at present no evidence on the question.

SUMMARY.

The antigenic complex of *Br. melitensis* exists in two states of aggregation which are distinguishable by serological as well as by physico-chemical methods. AP, the more highly dispersed complex, contains both the M and the A antigens of Wilson and Miles (1932). The more highly aggregated complex [PLAPS] appears to contain antigenic determinants of rough *Br. melitensis* as well, but the physical heterogeneity and instability of [PLAPS] solutions make their serological definition difficult.

Solutions of [PLAPS] show anisotropy of flow, and the implied polarity of the component particles is paralleled by the formation of oriented antigen-antibody complexes when [PLAPS] is mixed with some specimens of specific antiserum.

The physical and serological effects of dispersing both types of antigen with sodium dodecyl sulphate are described.

We are indebted to E. M. Miles for help with the serological tests ; and to Imperial Chemical Industries for the specimen of sodium dodecyl sulphate.

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CARCINOGENIC AGENTS PRESENT IN THE ATMOSPHERE AND INCIDENCE OF PRIMARY LUNG TUMOURS IN MICE.

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In previous papers results were published for experiments in which mice were exposed to road dust containing tar (Campbell, 1934), and also to road dust from which the tar had been extracted by benzene (Campbell, 1937*a*). It was demonstrated how such dusts containing tar readily produce cancer of the skin in mice, and also how road dusts—with and without tar—increase the incidence of lung tumours in mice, the tar dust being the more potent. Results have also been published for effects of exhaust gases from internal combustion engines and also for effects of tobacco smoke (Campbell, 1936). Recently, thanks to the courtesy of the London Passenger Transport Board, an opportunity was granted to test the effects of the soot from the exhaust pipe of internal combustion engines burning heavy oils. The results of these experiments are given herein, together with results obtained when using soot from a domestic chimney ventilating an ordinary coal fire.

Various tars extracted from all the above substances have been tested for carcinogenic agents using the simple skin-painting technique on mice.

METHODS.

For the inhalation of the soots the same large respiratory chambers were employed as in the previous researches (Campbell, 1934). Batches of 150 mice, half males and half females aged 3 months, were used, 75 of them as controls, while the other 75 were exposed to a moderate cloud of soot inside the chamber once an hour for 6 hours on 5 days of each week for a year. The soot came into contact also with the fur, and was not rubbed in or dusted off except by the mice themselves.

The mice were separated into groups, usually 8 or 10, in boxes with perforated zinc lids (area 28 $\times$ 21 sq. cm.); the holes in the lid occupied approximately half of this surface. A foot-bellows was employed to blow the soot into the chamber from a bottle, and the air blown in also ventilated the chamber sufficiently once an hour. The chamber was large enough, with 75 mice inside it, to prevent any undue accumulation in one hour of carbon dioxide or undue fall of oxygen percentage. A large fan stirred up the air well and was kept running except for 10 minutes every hour, when the cloud of soot was present.

The dusted mice were removed every evening from the boxes in which they had remained during exposure to the soot, and were placed in clean boxes with

bedding of sawdust and supply of water and food. The latter consisted of moistened stale brown bread, milk, cod-liver oil, oats, wheat germ, hemp, canary seed, linseed and sprouted oats.

RESULTS.

Results for Inhalation of Soot.

Chimney soot.—The analytical methods used to determine the composition of this soot were similar to those used previously. The main results are given in Table I. There was about 11·8 per cent. of benzene-soluble matter, probably all tar. The amount of ash varied considerably in different samples, forming from 14 to 36 per cent. of the total. The ash came from the brickwork and cement of the chimney, while some of the iron was derived from the metal-work. The soot also contained sulphur and was acid in reaction. The average amount of soot falling inside each box *per diem* was about 0·150 g., so that each mouse could come into contact with, on an average, about 15 mg. of soot or 2 mg. of tar *per diem*. This is much the same quantity as when dust from tarred roads was employed (1934). Much soot was observed in the lungs.

TABLE I.—*Composition of Chimney Soot. Percentages of Total Soot.*

Sample I :

Combustible matter	63·7	Ash	36·3
Carbon (by difference)	51·9	SiO_3	9·8
" Tar " (benzene soluble)	11·8	Al_2O_3	9·8
		Fe_2O_3	8·9
		CaO	5·4
		MgO	0·7

Sample II :

Combustible matter	85·86	Ash	14·14

Sample III :

Sulphur content	11·8 per cent. of total.
Total acidity	35·3 g. KOH/100 g.
Water-soluble acidity	7·6 g. ,,
Organic acidity (by difference)	27·7 g. ,,

The death-rate and changes in body-weight (Table II) are within the limits for those observed with control mice. The data for the latter are therefore not included here. Full details for large numbers of controls have already been recorded in the previous publications. As the mice were about 90 days old at the commencement of exposure to soot, the correct age is obtained by adding 90 days to the durations given in Table II.

Although the soot came into contact with the skin it did not cause warts or cancer. Also the incidence of lung tumours is not high when compared with the results for some of the batches of controls (see Table V). These points will be discussed later.

TABLE II.—*Mice Exposed to Chimney Soot and Exhaust Soot (Inhalation Experiment).*

Number of days of experiment.	Number of mice alive.		Weight (g.) of living mice.		Animals with lung tumours.			
					Chimney soot.		Exhaust soot.	
	Chimney soot.	Exhaust soot.	Chimney soot.	Exhaust soot.	Simple.	Malignant.	Simple.	Malignant.
0	74	75	20·4	17·7	0	0	0	0
100	68	58	25·0	25·1	0	0	0	0
200	60	57	28·6	30·2	0	0	0	0
300	38	54	30·0	31·4	2	0	0	0
400	32	42	27·6	34·0	2	1	1	0
500	17	30	28·7	32·5	3	2	1	0
600	11	9	27·3	32·0	4	4	6	9 (1 metastasis)
700	3	4	24·3	32·2	6	4	6	11
776	3 K*	2	23·0	30·0	8	4	6	13

* K = Killed.

Exhaust soot.—Some data dealing with the composition of this soot obtained from internal combustion engines burning heavy oils are recorded in Table III. The benzene-soluble matter was only about 2 per cent. of the total, while the acetone-soluble substance constituted about 13 per cent. The ash varied a good deal in absolute amount (1·5–7 per cent.) in various samples, being composed mainly of iron from the surface of the exhaust pipe. There was also 3·5 per cent. of sulphur present and the soot was acid.

TABLE III.—*Composition of Exhaust Soot.*

Sulphur content (by combustion in bomb calorimeter)	3·52 per cent. of total.
Total acidity	7·0 g. KOH/100 g.
Water-soluble acidity	5·1 g. ,,
Organic acidity (by difference)	1·9 g. ,,
Benzene-soluble extract	1·87 per cent. of total soot.
Acetone- ,, ,,	11·10 ,, ,, ,,
Alcohol- ,, ,,	4·26 ,, ,, ,,
(These extractions followed one another in the order mentioned. The benzene extract was soluble mainly in acetone, and the acetone extract was soluble mainly in alcohol.)	
Ash (mainly Fe)	1·71–7·4 per cent. of total.
Carbon (by difference)	73·0 ,, ,,

The death-rate was lower than that with the chimney soot (Table II), that is, more of the mice were alive between the 300th and 500th days of the experiment, but the death-rate was well within the limits for controls. The mice were heavier than those exposed to the chimney soot, and the latter—probably owing to greater acidity—appeared to affect the general health somewhat more than did the exhaust soot. None of the mice dusted with the exhaust soot developed warts or cancer of the skin. The total amount of soot entering the atmosphere of each of the mouse boxes *per diem* was similar to that for the chimney soot. The lungs of the mice contained much soot.

The incidence for primary lung tumours was on the high side, since 19 out of 57, or 33 per cent., of the mice living to 10 months of age or longer developed tumours (Tables II and V); 13 of these were malignant, and 1 of them gave rise to a metastasis or extension into a tracheo-bronchial lymph-gland.

Tar-painting Experiments.

Tars from road dusts.—Benzene was used to extract the tar from two samples of dust obtained from a tarred road and from one sample of dust collected from the surface of a road covered with "tar-mac". The benzene was then distilled off, leaving a residue with the consistence of thin treacle. This was painted twice a week on the skin of 24 mice at the middle of the back, just behind the shoulders. A road top-dressing of tarry matter was also tested in the same way. The painting was continued for about 6–7 months, and all these four samples of road tar contained an active carcinogenic agent, warts appearing on the painted site of the skin of many of the mice within 6 months

of commencement of the painting (Table IV, *a*, *b*, *c*, *d*). The third column of this table gives the number of mice with warts of the skin as the numerator, the denominator being the total number of mice still alive after six months' painting. At this time there was a total of 87 mice alive in the 4 batches, and 65 of these revealed typical cancer of the skin at death. In 16 of these cases the skin cancer gave origin to metastases in the lungs. There were in addition 10 of the total 87 mice with primary lung tumours, half of these being of the malignant type. This is not an abnormally high incidence for primary lung tumours, since the control mice of the same strain often show a similar incidence. Murphy and Sturm (1925) observed primary lung adenoma in up to 78 per cent. of fairly young mice, of which the skin had been painted with tar on various different areas to prevent irritation and development of skin cancer. Their technique thus differs from the present technique. That the presence of skin cancer may not inhibit development of primary lung tumours was proved in one of the earlier researches (Campbell, 1934) in which mice were dusted (and not painted at all) with tar dust ; these mice developed cancer of the skin, and at the same time showed an abnormally high incidence of primary tumours of the lung. Watson and Mellanby (1930) give similar evidence from painting experiments.

"Tar" from cigarette smoke.—Cigarettes were smoked artificially (Campbell, 1936) and the hot smoke was passed through a cool glass tube, in a side bulb of which some of the substance of the smoke condensed to form a dark brown liquid. This was concentrated and painted on the skin of 23 mice in the usual way, twice a week for 20 months. In one case a typical epithelioma developed (Table IV, *e*), the wart being first noticed after 16 months of painting. In another of these mice which had been painted for 20 months the painted area of skin at death was much thickened, and on section revealed the typical hyperplasia, which represents the first stage of cancer reaction to tar painting. The tarry matter used had not been exposed to a temperature higher than that produced in a cigarette smoked in the ordinary way. Compared with the road tar this tobacco " tar " is certainly not very active, but it apparently may give rise to cancer of the skin after prolonged application.

Seven, or 35 per cent., of the 20 mice living 10 months or longer and painted with cigarette " tar " exhibited primary carcinoma of the lung. Twelve of these mice lived for 2 years or longer—that is, to an age favourable to the development of lung tumours.

Benzene extract of cardboard.—In the course of some experiments it was observed that certain samples of cardboard contained a binding material of a tarry nature. This was extracted from the cardboard and painted on the skin of 24 mice in the usual way twice a week for 12 months. No warts or cancer of the skin developed, but 10, or about 50 per cent., of the mice surviving for 10 months possessed at death primary lung tumours (Table IV, *f*), half of them being malignant. One of them gave rise to a metastasis or direct extension into the tracheo-bronchial lymph-glands and parietal pleura. This is a high incidence of primary lung tumours for our mice, and it is possible that some of the dried tarry material may have been inhaled during attempts to remove it and acted as an irritant.

Tar from exhaust soot.—The soot obtained from the exhaust pipe of engines

TABLE IV.—*Tar-painting Experiments; Warts and Cancer of Skin; Tumours of Lung.*

"Tar" used.	Duration of painting (months).	Number of mice with warts.		Number of mice.		Number of mice with primary lung tumours.		Number of mice surviving.		
		After six months' painting.	At death.	With cancer of skin at death.	With metastases from skin.	Simple.	Malignant.	10 months.	18 months.	24 months.
(*a*) B.E.* road dust I	7·3	16/20	16	16	6 (in lung)	0	0	18	1	0
(*b*) B.E. road dust II	7·3	3/22	11	10	1 (,,)	2	4	21	12	3
(*c*) B.E. "tar-mac"	6·5	16/23	20	19	5 (,,)	0	0	19	4	0
(*d*) Road top-dressing	6·5	15/22	20	20	4 (,,)	3	1	21	5	0
(*e*) Tobacco "distillate"	20·0	0/20	2	1	0	0	7	20	17	12
(*f*) B.E. cardboard	12·0	0/20	0	0	0	5	5 (? 1 with metastasis)	20	12	2
(*g*) Acetone E. exhaust soot	12·3	0/21	0	0	0	1	1	20	13	3
(*h*) B.E. exhaust soot	6·6	0/22	0	0	0	1	2	22	8	5
(*i*) B.E. chimney soot	6·0	0/23	9	7	1 (in liver)	2	2	21	5	0

* B.E. = Benzene extract.

burning heavy oil was submitted to extraction, both by acetone (Table IV, *g*) and by benzene (Table IV, *h*). Both extracts, when concentrated, were painted on the skin of 24 mice twice a week, the former extract for a period of 12 months and the latter for a period of 6 months. In neither case were warts nor cancer of the skin produced, so that no active carcinogenic agent appeared to be present.

Only 2 of the mice painted with the acetone extract developed primary lung tumours, one of which was malignant, while 3 of the mice painted with the benzene extract of the exhaust soot developed tumours of the lung, 2 of them being malignant.

Tar from chimney soot.—This tar was extracted by benzene and painted on the skin of 24 mice twice a week for about 6 months. None of the mice developed warts within the 6 months of painting, but 9 of them did so later on, and 7 of these developed typical carcinoma of the skin, 1 of which produced a metastasis in the liver (Table IV, *i*). This confirms the earlier work of Passey and co-workers (1922, 1925) as to the experimental production of cancer by agents in coal soot.

In the present experiment, 4, or about 19 per cent., of the 21 mice living 10 months or longer developed primary adenoma of the lung, 2 of them being malignant.

DISCUSSION.

Carcinogenic Agents in the Atmospheric Air.

The results for the skin-painting experiments (Table IV, *a*, *b*, *c*, *d*) indicate clearly that the most potent carcinogenic agents likely to pollute the atmosphere are carried in the dusts from tarred roads. Not only may the presence of a carcinogenic agent be proved by tar-painting experiments, but also by merely dusting the mice with the road dust (Campbell, 1934). The resulting skin cancer is typical of tar, and also is capable of giving rise to metastasis in the lungs. It is possible that the inorganic substances present in the road dust aid the tar in the production of the skin cancer in these dusting experiments.

The tar present in chimney soot is also potent as tested by skin-painting experiments (Table IV, *i*), but this soot does not appear to be so dangerous as the road tar dust, because the latter does and the former does not cause cancer of the skin when merely dusted on the mice. Chimney soot produces cancer in man when rubbed into the skin from the soiled clothes of chimney-sweepers. It would appear in the case of the dusted mice that the carbon in the soot dusted on the skin antagonizes the action of the tar, and it is probable that it does so in man to some extent ; that is, the chimney-sweeper's cancer would be much more prevalent if the carbon were not there too.

The soot from the exhaust pipes of engines burning heavy oil does not contain an active carcinogenic agent. Of course all vehicles stir up the road dust with its tar, and the chief danger seems to lie in this finely divided and continually stirred-up tar dust.

The tarry matter obtained from cigarettes as usually smoked does not appear to be very dangerous, but evidence is obtained (Table IV, *e*) that it can produce typical carcinoma of the skin after prolonged application. Schürch

and Winterstein (1935) obtained some similar evidence under certain conditions, and Roffo (1937) has produced cancer of the skin of the ear of the rabbit by painting for 10 months with tars derived from distillation at 120°–350° C. and from the residue. He states that 90 per cent. of the cancers of the mouth and respiratory tract occur in heavy smokers.

Atmospheric Pollution and Primary Lung Tumours.

The results to date for incidence of primary lung tumours of control mice and mice exposed to increased atmospheric pollutions are given in Table V. In this table only the mice living 10 months or longer are considered. Mice of the same mixed stock were used for controls and for the experiments, and each batch of results represents more than 50 mice. It was obvious among the controls themselves that age is a factor in the development of lung tumours, these being rare before 12 months of age. In these controls a causal factor may be some irritant carried via the air breathed by the mice. Other factors have already been discussed (Campbell, 1937*b*).

Of the inhalation experiments the gases—without the soot—from the exhausts of internal combustion engines are not very potent in producing primary lung tumours, nor is chimney soot. Chimney-sweepers show some slight increase (Kenneway and Kenneway, 1936), and Seelig and Benignus (1936), using coal smoke soot as bedding for mice, obtained some increase in incidence of carcinoma of the lung. In another experiment (1938), using a cancer-resistant strain of mice and a 10 per cent. mixture of a carcinogenic tar in lamp-black, they failed to induce cancer of the lung, and consider that the softer basis might explain the difference, the coal soot being more irritating than the lamp-black. This factor of irritation was regarded as important in the experiments with road dust from which the tar—or most of it—had been removed (Campbell, 1937*a*, *b*, 1938). The inorganic constituents are under suspicion as factors in the production of lung tumours and also of skin cancer in the mice merely dusted with dust from tarred roads (Campbell, 1934). The carbon in the soot may be less irritating than the inorganic constituents in the road dust, and the incidence of lung carcinoma in chimney-sweepers might be higher than it is were it not for the presence of this carbon. Silica and iron of the road dust are regarded as possible irritants, and are being tested. Already there is some evidence that precipitated silica may produce cancer of the lung of mice. This experiment is being continued. Silica and iron are also present in the atmosphere in the vicinity of metal-grinders, who show a relatively high incidence of carcinoma of the lung (Kenneway and Kenneway, 1936). Turner and Grace (1938) also draw attention to the high mortality from cancer of the respiratory tract in engineers, foundry workers and grinders. They say this is seen in no other occupational group. We received a sample of dust from the neighbourhood of the mines in Jachymov, Czechoslovakia, where there is a high incidence of lung tumours. Silica and iron are among the inorganic constituents in this dust and may be factors in the development of the carcinoma of the lung, although the radium and arsenic of the mines are usually considered the most active agents in this case. The literature on this point has already been summarized (Campbell, 1937*b*; Döhnert, 1938). In all our

experiments with mice the road dust containing tar produced the highest incidence of lung tumours (Table V), and it is concluded that the inorganic salts aid this tar in its carcinogenic action.

TABLE V.—*Results for Primary Lung Tumours of Mice Living 10 Months or Longer (Inhalation Experiments).*

Condition of mice.	Percentage of mice with lung tumours.	Greatest age of mice (days).
Normal controls (A)	8	664
,, ,, (B)	8	705
,, ,, (C)	13	841
,, ,, (D)	14	849
,, ,, (E)	20	919
,, ,, (F)	20	912
Motor exhaust gas (1)	18	849
,, ,, ,, (2)	26	841
Chimney soot	20	860
Mice dusted (road dust) one week only	30	912
Cigarette smoke	30	919
Exhaust soot	33	866
Road dust — tar	45	912
,, + tar + CO	47	705
,, + tar	74	664

We also obtained a slight increase in incidence of mouse lung tumours with the soot from internal combustion engines burning heavy oil (Table V). This soot, as already indicated, contained some iron and sulphur, and was acid in reaction. It is possible that one or all of these, or some other factor, acted as irritant to the lung, seeing that the tar from this soot when painted on the skin for 6 months and longer did not produce cancer. There is thus more evidence for production of lung tumour by irritation independent of the presence of any special carcinogenic substance. Holmes (1938) records 4 cases (ages 30, 39, 41 and 48 years) of lung carcinoma in men employed on work with internal combustion engines. Alexander (1937) gives details of a lorry worker aged only 19 years who died of lung cancer. The soot from the exhaust pipe may have played some part, but it is probable that all these patients had been exposed also to much irritation, for example, from fine dust from tarred roads. This tar dust seems to be the most potent agent in our experiments.

Cigarette smoke also gives an incidence of lung tumours in mice on the high side (Table V). We have shown above that the tar from such smoke can produce cancer of the skin after prolonged application, while Roffo (1937) produced cancer of the skin of the ear of rabbits with similar tar.

There has been some criticism that lung tumours of mice are not malignant. This, we think, is due to the fact that the simple adenoma is usually the more prevalent in mice under normal conditions, and particularly in the younger mice —12–18 months. In our dusting experiments we have certainly obtained typical malignant tumours of the mouse's lung, some of them accompanied by typical

metastases (Campbell, 1937*a*). Further examples of metastases have been observed more recently. These malignant tumours invade and destroy other tissues.

SUMMARY AND CONCLUSIONS.

1. Tarry matters from the following have been tested for carcinogenic potency, using the mouse-skin test: Dusts from tarred roads; cigarette smoke; chimney soot; soot from exhausts of internal combustion engines burning heavy oil; a binding material in cardboard.

2. As proved by the production of skin cancer, the tarry matter from tarred roads contains potent carcinogenic agents as does chimney soot, but under ordinary conditions the effect of the latter in its action on the skin seems to be mitigated somewhat by the presence of the carbon of the soot, while the effect of the former seems to be aided by the presence of inorganic constituents.

3. Cigarette smoke, under ordinary circumstances, gives rise to tarry matter which may occasionally produce hyperplasia and cancer of the skin after prolonged application.

4. Tarry matters from the motor-exhaust soot and from the cardboard tested do not produce cancer when painted on the skin of mice.

5. Exhaust soot suspended in the atmosphere slightly increases the incidence of lung tumours in mice, while chimney soot does not produce any noticeable increase. The dust from tarred roads appears to be the most dangerous source of carcinogenic agents; when inhaled it gives the highest incidence of lung tumours in mice. It is considered that here again the inorganic constituents in this dust (for example, silica, iron) enhance or aid the effect of the tar, while, on the other hand, the carbon of soot tends to mitigate any effects of the tar present in producing tumours of the lungs. Irritation without inflammation appears to play a part in genesis of these tumours both in control and experimental mice.

6. Although some primary lung tumours in mice are simple adenomata, other tumours reveal characters of typical malignancy with production sometimes of metastases. Further examples of metastases have been obtained.

I am indebted to the London Passenger Transport Board for providing the sample of exhaust soot from engines burning heavy oil and also for some data *re* analyses. Thanks are also due to the mining authorities in Jachymov, Czechoslovakia, for the sample of dust from the neighbourhood of their mines; also to Mr. W. W. Starling for assistance with some of the extractions and analyses; to my technical assistant, Mr. C. Pergande, for much help throughout this research; and to Mr. F. J. Higginson for cutting and staining the sections.

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THE ACTION OF PROTEOLYTIC ENZYMES ON THE ANTITOXINS AND PROTEINS IN IMMUNE SERA.

I.—TRUE DIGESTION OF THE PROTEINS.

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FOLLOWING the discovery of diphtheria antitoxin by Behring (1890), many workers attempted to isolate the specific antibody and to determine its chemical properties. The activity was found to be associated with the proteins, and in particular with the water-soluble globulin known as pseudoglobulin. Most of the early work has been reviewed by Dean (1908) and later by Marrack (1938), and will not be dealt with here.

The usual method of fractionation using a reversible precipitant such as ammonium sulphate or sodium sulphate removes fibrinogen, part of the euglobulin and the albumin, the antitoxin being mainly found in the pseudoglobulin.* A single fractionation does not give a pure pseudoglobulin, but one contaminated with some euglobulin and albumin, and also with lipoid material, with the result that the product shows change with age, and usually there is separation of a precipitate of protein or lipoid. This led to special methods of concentration involving repeated fractionation with salts, or dialysis to a "salt-free" condition followed by extensive dilution and adjustment of the pH to the isoelectric point of the water-insoluble protein-lipoid complex. These extensions of the method, although yielding a product of greater stability and improved condition, did very little to increase the actual purity of the product in terms of units of antitoxin per g. of protein. The small increase in real purity is one of the greatest objections to the usual methods employed

for serum concentration since, as is well known, there is a marked increase in the pseudoglobulin fraction as a percentage of the total protein in the immunized animal, and this increase, together with unavoidable losses of antitoxin, limits the increase in purity to about 1·5 times that of the starting plasma or serum. Most of the increased value in terms of units/ml. arises from the fact that, as usually prepared, the concentrated serum contains about 15 per cent. of pseudoglobulin. This argument does not obscure the clinical advantage arising from the elimination of albumin and euglobulin.

The small increase in real " purity " obtained with salting-out methods is due to the fact that the antitoxin is not specifically precipitated by these salts. For example, it can be shown that if the toxin (*e. g.* diphtheria toxin for diphtheria antitoxin) is used as a specific precipitant for its antitoxin, only about 10–20 per cent. of the total protein of the concentrated material is " antitoxic protein ", the other being non-specific pseudoglobulin having the same solubility in the salt solution employed for concentration.

It was natural that the action of enzymes on antitoxin should have been studied. Pick (1902), Mellanby (1908), and Schmidt and Tuljtschinskaya (1931–2) all found that antibodies were rapidly destroyed by pepsin and less rapidly by trypsin. More recently Rosenheim (1937) has studied the action of pepsin, trypsin and activated papain on agglutinins produced in horses or rabbits after immunization to *B. typhosus*. She found that in the early stages of immunization both the flagellar (H) and somatic (O) agglutinins were easily destroyed by these enzymes, although after several immunizations the H agglutinins became more resistant to pepsin and trypsin, but not to activated papain.

The attempts, made many years ago, to use enzyme methods for the purification of antitoxins were handicapped for two main reasons: (*a*) lack of control of factors, such as the reaction (pH) of the digestion mixtures, and (*b*) the lack of a simple method for estimating the antitoxin content during digestion and isolation of fractions. The substitution of an *in vitro* method (Ramon flocculation) for the *in vivo* methods (various forms of animal tests) has led to an enormous increase in the amount of experimental work carried out on diphtheria toxin and antitoxin. The difficulties outlined probably explain the fact that although a patent was taken out in this country in 1902 (Imray, 1902) for a method for purifying antitoxins by digestion with either pepsin or trypsin, the method does not appear to have been used commercially on any scale. Nevertheless, the methods described in the patent have been used by the writer, and have yielded a product having a greater purity than can be obtained from the usual " salting-out " methods using ammonium sulphate for fractionation. More recently, Parfentjev has obtained two patents relating to the purification of antitoxic sera by means of enzyme digestion. The first of these (U.S. Patent No. 2065196) deals with the digestion of unwanted protein by means of pepsin within a restricted pH range, and the second (U.S. Patent No. 2123198) with the adsorption of some non-antitoxic undigested proteins which are present in the digest product. Although work had been carried out at these laboratories by Mellanby (1908) and some years ago by the writer, the results obtained were not very promising. The difficulties were those already mentioned.

The publication of a reference by the Council of Pharmacy N.N.R. (*Journ. Amer. Med. Assoc.*, 105, 883, 1935) to an American patent on a new method of concentration redirected attention to the possibilities of enzyme action on antitoxin, and a good deal of credit is due to Parfentjev for having refocused attention on this interesting problem.

In a recent paper (Pope, 1938) I have described a method which involves slight modification of the antitoxic pseudoglobulin by means of enzyme action, followed by critical heat denaturation of non-antitoxic proteins as a process for the purification of antitoxins. In the course of the work on the action of enzymes on antitoxin, a study of the digestion of protein to a non-protein state was made. As certain new points have emerged from this work it is proposed to deal with "true digestion" in this paper, and to leave the special "heat denaturation" process for a later paper.

EXPERIMENTAL.

Methods.

Estimation of the antitoxin.

Owing to the easy methods of testing which are available for diphtheria antitoxin this material was used for most of the experimental work. Generally, tests were made by the flocculation method and were confirmed by animal tests. It is a point of interest that antitoxins suitably treated with enzymes show a much reduced time of flocculation (Pope and Healey, 1938; Hewitt, 1938), and an explanation for this will be advanced later. In most cases flocculation tests were accurate to within 5 per cent.

Estimation of protein.

The estimations were all made with a Zeiss dipping refractometer, using a heat coagulation method which we have used at these laboratories for many years. Since the method is almost identical with that recently published by Siebenmann (1937), it will not be given in detail. The constant $a = 0{\cdot}00185$ has been used for calculating the protein, both for the original protein and for that remaining after enzyme action. Cross-checks have also been made in the usual manner from nitrogen determinations (total nitrogen-non-protein nitrogen). The refractometric protein estimations usually agreed to within 1 per cent.

The purity of the antitoxin.

At present all the available evidence suggests that antitoxins are modified globulins, and their purity is, therefore, best expressed as the "number of units of antitoxin per g. of protein". From determinations which have been made on floccules produced by the specific precipitation of antitoxin with toxin, figures varying from about 60,000 to 80,000 units per g. of protein have been obtained (Ramon, 1923; Marrack and Smith, 1930; and Pappenheimer and Robinson, 1937). When the antitoxin has been modified by enzyme action there appears to be less protein associated with the antitoxin, as shown by Pappenheimer and Robinson (1937) and Pope (1938). Antitoxin of such

purity that all the protein present was specifically precipitated by toxin was reported by Pope (1938, 1939), and this material contained 135,000 units per g. of heat-coagulable protein. These figures give some indication of the probable purities of the unmodified and modified forms of diphtheria antitoxin. Since both the protein and antitoxin contents can be determined accurately on the original serum and on various fractions, this figure is the best guide to increased purity as a result of any treatment. The change is conveniently expressed in the form of a ratio figure (R value). For example, a serum may contain 1000 units of antitoxin per ml. and 9 per cent protein, giving a value of 11,100 units/g. protein. As a result of purification, suppose 85 per cent. of the protein is removed for a loss of 30 per cent. of the antitoxin, the new figure becomes 51,800 units/g. protein. This can be expressed in the form—

$$\frac{\text{Percentage antitoxin left}}{\text{Percentage protein left}} = \frac{70 \text{ per cent.}}{15 \text{ per cent.}} = 4{\cdot}65 \text{ R value,}$$

or—

$$\frac{\text{Final units/g. protein}}{\text{Original units/g. protein}} = \frac{51{,}800}{11{,}100} = 4{\cdot}65 \text{ R value.}$$

Enzymes used.

All the enzymes given below have been studied for their action on antitoxic sera. Pepsin, trypsin and activated papain all produce fairly extensive true hydrolysis of the serum proteins, while fibrinolysin and the proteolytic enzyme present in maltin only produce minor changes. The conditions for the use of each enzyme system vary, and although most of the results in this paper will deal with pepsin, the conditions for the other enzymes will be considered.

Pepsin was used in the forms commercially available without any purification. The activity of the products used varied from B.P.1/2000 to 1/10,000, but no marked differences were observed in the action of pepsins of varying strength or from different sources.

Trypsin extracted from ox pancreas by Cole's method (Cole, 1916) was used for some experiments. For others, acid-treated trypsin (containing chiefly the proteinase system) was used.

Papain was used in the usual powder form. Several activators were tested; thioglycollic acid proved to be one of the most satisfactory.

Maltin (diastase) Schering-Kahlbaum.

In addition to diastase this enzyme preparation contains a proteolytic enzyme system.

Fibrinolysin.

Fibrin clots, obtained by adding calcium chloride to oxalated horse-plasma and shaking until clotting took place, were well washed and then extracted with dilute acetic acid. As an alternative method, the clot was allowed to autolyse in a buffer solution at pH 8, containing 0·2 per cent. tricresol, at 37° C. Both these products contained active enzymes.

The Effect of pH during Digestion.

The first point investigated was the influence of pH on the digestion of protein and the stability of the antitoxin. In order to reduce any complications which might arise from the presence of known non-antitoxic proteins, such as fibrinogen, euglobulin or albumin, a pseudoglobulin solution purified in the usual manner with ammonium sulphate was used. The protein present in the solutions, after pH adjustment, was 1·58 per cent. All pH measurements were made with a glass electrode, standardized with buffer solutions of known value. The pepsin used had a value of 1/3000 B.P., and was added in a ratio of 1 part of pepsin to 200 parts of protein. Mixtures at varying pH values were heated at 35° C. for 24 hours, after which time their protein and antitoxin contents were determined. The collected results for a number of such experiments are shown in Table I. At pH values less than 2·9 marked destruction of antitoxin took place. The figures also show that for a pseudoglobulin solution there was little evidence for digestion of protein without a parallel loss of antitoxin.

TABLE I.—*Showing the Stability of Antitoxin and Digestion of Protein at Varying pH Values. Pepsin Ratio* 1/200. *Temperature* 35° *C. for* 24 *Hours. The Antitoxin Used had been Purified by the Ammonium Sulphate Process and Consisted of the Pseudoglobulin Fraction.*

pH.	Percentage of original protein undigested.	Percentage of original antitoxin left.
1·3	13·5	0
1·85	18·0	0
2·05	18·0	0
2·15	19·0	0
2·32	31·0	0·14–1·4
2·55	47·0	7–14
2·85	59·0	43–57
2·90	58·0	63
3·05	67·0	70
4·4	91·0	100
5·3	95·0	100

All antitoxin tests for this experiment were made *in vivo*.

On the same material other observations were made at room temperature over a period of 7 days. The results, given in Table II, show once again that the loss of antitoxin was very great at pH values less than 2·9, and that in the controls with no pepsin, a similar loss took place at these low pH values. The destruction of antitoxic activity was not therefore due to the action of pepsin under these conditions.

TABLE II.—*Showing the Results after 7 Days at Room Temperature. Conditions otherwise as in Table I.*

pH.	Controls without pepsin (antitoxin left) (per cent.).	With pepsin.	
		Antitoxin left (per cent.).	Protein undigested (per cent.).
1·38	about 10	0	10
1·88	,, 10	0	30
2·90	57	76	32
4·40	100	92	97
5·15	100	100	100

Since these experiments showed no clear evidence that pepsin in low concentrations could cause digestion of protein in this pseudoglobulin solution without simultaneous destruction of antitoxin, the effect of increased concentrations of pepsin was studied. The pepsin-protein ratio was therefore increased from 1–200 to 1–40 and 1–20, and its action studied at pH values from 3–5. Table III shows the results obtained after 24 hours' digestion at 37° C. The only significant digestion of protein without proportionate antitoxin loss was at pH 3. These samples at pH 3 were again examined after a further 24 hours at 37° C. (48 hours total). For the pepsin-protein ratio of 1–40, 40 per cent. of the protein was undigested and 61 per cent. of the antitoxin remained, giving an increased purity of 61/40, = 1·5 times. At the pepsin-protein ratio of 1–20, 27 per cent. of the protein was undigested and 61 per cent. of the antitoxin remained, giving an increased purity of 61/27, = 2·26 times. It was therefore clear that a fairly large concentration of pepsin was necessary to secure an increase in purity of this antitoxic material. For a loss of about 40 per cent. of the antitoxin, this material, already purified by the ammonium sulphate process, showed a further increase in purity of 2·26 times.

TABLE III.—*Showing the Protein and Antitoxin Changes after 24 Hours at 37° C. for Two Ratios of Pepsin/Protein at Varying pH Values. Material: Antitoxic Pseudoglobulin Solution.*

pH.	Ratio pepsin/protein.	Percentage protein left.	Percentage antitoxin left.
3·05	1/40	49	61
3·52	1/40	68	65
3·75	1/40	74	72
4·14	1/40	82	80
4·50	1/40	84	80
4·93	1/40	90	81
3·00	1/20	39	62
3·45	1/20	61	71
3·78	1/20	70	75
4·14	1/20	78	75–80
4·51	1/20	87	81
4·91	1/20	88	85

On the basis of these results an attempt was made to treat antitoxic plasma by a digestion method. Plasma containing 690 units per ml. and 9·79 per cent. protein was diluted with a half volume of water, adjusted to pH 3 with hydrochloric acid and pepsin added (ratio pepsin-protein, 1–20). The solution was digested at 36° C., and samples examined at intervals. Table IV shows that there was a marked loss of antitoxin without any preferential digestion of proteins. At the end of 5 hours the addition of extra pepsin failed to alter the rate at which protein was digesting. The digestion-rate was increased by adjusting the pH back to 2·9, but this caused a large loss of antitoxin.

TABLE IV.—*Showing the Changes in Protein and Antitoxin on Digestion at pH 3 with Pepsin. Material: Antitoxic Plasma.*

Time at 36° C.	pH.	Protein left (per cent.).	Antitoxin left (per cent.).
0 hours	3·0	100	100
1 hour	3·25	55	60
2 hours	3·28	49	57
3 ,,	3·30	48	54
4 ,,	..	46	54
5 ,,	..	44	51

Some additional experiments were made with this antitoxic plasma at less acid pH values. At a pH value of 4·2, after 68 hours' digestion at 37° C., 47·5 per cent. of the protein remained undigested and 78 per cent. of the antitoxin was left. The units per g. of protein had increased from 7200 to 11,800, giving a ratio of 1·64 times increased purity. This is about the order of increased purity which could have been obtained by ammonium sulphate fractionation, and at this stage the peptic digestion showed no advantage

TABLE V.—*Showing Protein and Antitoxin Changes in Samples A, B and C at 37° C. See text for details.*

Time at 37° C.		A. pH 4·47.	B. pH 4·2.	C. pH 4·0.
1 day	Antitoxin left	80%	76%	70%
	Protein left	45%	40%	30%
	Units per g.	12,700	13,900	16,700
	Ratio (increased purity)	1·76	1·93	2·32
2 days	Antitoxin left	79%	76·5%	70%
	Protein left	42·5%	36·8%	26·0%
	Units per g.	13,400	15,000	19,300
	Ratio (increased purity)	1·86	2·08	2·68
10 days	Antitoxin left	70%	66%	53%
	Protein left	32·8%	23·6%	15·4%
	Units per g.	14,800	19,500	23,800
	Ratio (increased purity)	2·05	2·7	3·3

over " salting-out " methods. The material after 68 hours' digestion was submitted to further treatment as follows :

The solution was divided into three equal volumes, of which—

(A) was left untreated as control (pH now 4·47) ;

(B) was readjusted back to pH 4·2 ;

(C) was readjusted back to pH 4·0.

Incubation was then continued at 37° C. for 10 days and the results given in Table V obtained. In solution (C), where the maximum increase in purity was obtained (3·3 times), the loss of antitoxin amounted to 47 per cent.

These results are typical of those obtained when an attempt was made to remove protein from antitoxic plasma of low to medium titre by means of peptic digestion methods.

The Effect of Different Pepsins.

Samples of pepsins from various sources having B.P. values varying from 1/2000 to 1/10,000 were compared on a sample of plasma, which after dilution with an equal volume of water was adjusted to pH 4. Sodium acetate (0·5 per cent.) was added as an additional buffer. The solution was divided into five parts, and the pepsin (A–E) added in a ratio of 1 part pepsin to 20 parts of protein. These solutions were all allowed to stand for 24 hours at room temperature before heating to 37° C., and at this stage all contained at least 90 per cent. of their original antitoxin. Table VI records the protein and antitoxin contents after 48, 120 and 216 hours at 37° C. None of the pepsins gave an R value figure greater than 2, and generally the increase in purity was of the same order as that obtainable by ammonium sulphate fractionation. One pepsin (A) appeared to give slightly better results than the others, and this one had the best B.P. value (1/10,000). This result suggested that a further increase in the amount of pepsin used might lead to improved results.

TABLE VI.—*Showing the Action of Various Pepsins.*

Time at 37° C.	Pepsin sample.	Protein left (per cent.).	Antitoxin left (per cent.).	R. value.	Units/g.
48 hours	A	46·5	79	1·7	..
	B	58·9	79	1·34	..
	C	71·2	79	1·11	..
	D	61·5	79	1·28	..
	E	63·4	79	1·24	..
120 ,,	A	40·3	77	1·92	10,850
	B	45·9	77	1·68	9,500
	C	47·2	77	1·63	9,300
	D	44·9	77	1·71	9,700
	E	45·1	77	1·70	9,650
216 ,,	A	34·6	66·5	1·92	11,000
	B	40·6	68·5	1·68	9,500
	C	41·6	70·0	1·68	9,550
	D	40·5	70·0	1·73	9,800
	E	40·6	68·5	1·69	9,550

The original plasma contained 5650 units per g. protein.

The digestion of high titre plasma.

The work recorded on plasma had been carried out on material of 500–600 units per ml., obtained from horses which had not responded too well to immunization. It was therefore decided to study the digestion of high-titre plasma, and at the same time investigate the advantage, if any, of using more pepsin.

Plasma containing 1500 units of antitoxin per ml. was diluted with half its volume of water and adjusted to pH 4·25. After subdivision into four equal volumes pepsin was added to give the following pepsin-protein ratios : (A) 1–10, (B) 2–10, (C) 3–10, (D) 4–10, and the solutions were then kept at room temperature for 24 hours before heating to 37° C. The results after 48 hours' digestion are shown in Table VII, from which it will be seen that protein digestion, antitoxin loss and increase in purity (R value) took place to about the same extent, irrespective of the amount of pepsin used. Moreover, with this high-titre plasma, protein digestion was more extensive than was obtained in the earlier experiments, and the antitoxin loss less, with the result that an increase of about four times in the purity was obtained.

TABLE VII.—*Showing the Action of High Concentration of Pepsin at pH 4·25 and 37° C. after 48 Hours.*

Ratio pepsin/protein.	Protein left (per cent.).	Antitoxin left (per cent).	Units/g.	Ratio.
1–10	20·8	82·5	36,800	3·96
2–10	19·6	82·5	39,000	4·22
3–10	19·5	81·0	38,500	4·12
4–10	20·0	82·5	38,400	4·12

Some other work carried out on another plasma of high titre (1760 units/ml.) showed this marked increase in purity. This plasma contained 0·4 per cent.

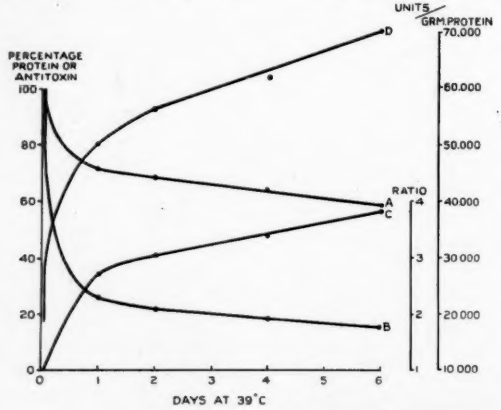

FIG. 1.—Digestion with pepsin. Curve { A. Antitoxin percentage remaining. B. Protein percentage remaining. C. Change in ratio. D. Units per grm. protein.

tricresol and was used undiluted. The pH in this case was adjusted to 4·2 with solid citric acid, and owing to the presence of tricresol and the fact that the plasma was used undiluted, the loss of antitoxin was high during the early part of the digestion. The data for this experiment given in Fig. 1 show the changes which took place during digestion at 39° C. Once again an increase in purity of nearly four times was obtained by digestion. A repeat experiment on this plasma, using more favourable conditions, gave a product which contained more than 80,000 units per g. of protein.

These results, obtained on plasma of medium or high titre, suggested that an investigation on plasma from horses showing slow or rapid immunization might throw light on the variation in digestion which had been noted.

Effect of the Stage of Horse Immunization.

It is well known that during immunization, changes take place in the serum protein content which may be out of all proportion to the antitoxin produced, and that such changes may involve both an increase in the total protein content, and an increase in the pseudoglobulin fraction as a percentage of the total protein. An attempt was therefore made to study the action of pepsin on plasma from horses which had given varying degrees of antitoxic response. Table VIII shows data for plasma selected for this purpose, and under the column headed " Toxoid " we show the impression of the antigenic value of the toxoid used, judged from its behaviour on a group of horses. Plasma from one horse (H. 6351) was obtained in an early stage of what was thought to be an immunization with a good toxoid, and hence has a low antitoxic value. For the purpose of comparison, plasma from one normal horse was included in the series.

TABLE VIII.—*Showing Details of Plasma Selected.*

Plasma.	Protein content (per cent.).	Antitoxin content. Units/ml.	Toxoid.*	Units/g. protein.
6193	10·8	362	Bad	3,330
6351	8·24	225	Good	2,730
6308	11·25	1180	,,	10,500
5984	9·6	480	Bad	5,000
Mixed plasma	9·25	1120	Good	12,200
6411	9·07	920	,,	10,150
6509	7·15	Normal horse	..	..

* See text.

Samples of plasma as detailed in Table VIII were adjusted to pH 4·4 with solid citric acid, control experiments having shown that in the pH range 3·5–4·5 citric acid gave the same digestion results as hydrochloric acid, but with less tendency for change in pH during digestion. The digestions were allowed to proceed for 7 days at 39° C., and measurements were made at intervals. The results of this experiment are recorded in Table IX. After 1 day's digestion there were considerable differences in the amount of antitoxin left, the amount of protein digested and the increased purity, although these samples had been

submitted, as far as possible, to identical treatment. These differences persisted throughout the experiment. Table X shows the actual amount of protein digested during 7 days at 39° C. for each plasma. The amounts varied from 6·8 to 8·55 g. of protein, and the percentage amounts from 71 to 95 per cent. The normal horse-plasma, which in 7 days lost 95 per cent. of its protein, lost 85 per cent. in the first 24 hours. By comparison the antitoxic plasmas lost from 54 to 70 per cent. of their protein in the same period of 24 hours. After 7 days' digestion the increased purity of the antitoxin varied from 2·35 to 4·5 times. This result is typical of the variation which may be encountered with plasma from individual horses.

TABLE IX.—*Showing the Action of Pepsin at pH 4·4 on Plasma from Different Horses.*

Horse.	Ratio after 1 day at 39° C.	Units per g. 1 day.	Ratio after 2 days at 39° C.	Units per g. 2 days.
6193	86/41·3 = 2·08*	7,000	83/35·3 = 2·35	7,850
6351	83/31·0 = 2·68	7,350	78·5/23·4 = 3·36	9,180
6308	71·5/39·0 = 1·92	20,000	74·5/32·1 = 2·32	24,400
5984	83/46·1 = 1·8	9,000	79/40·6 = 1·94	9,750
Mixed plasma	62·5/26·1 = 2·4	29,000	60·6/20·9 = 2·9	35,100
6411	74/37·0 = 2·0	19,000	67·5/28·4 = 2·38	24,100

Horse.	Ratio after 3 days at 39° C.	Units per g. 3 days.	Ratio after 7 days at 39° C.	Units per g. 7 days.
6193	79·4/31·1 = 2·55	8,550	70·5/25·4 = 2·78	9,250
6351	72/19·3 = 3·73	10,200	60/13·3 = 4·5	12,300
6308	74/29·7 = 2·51	26,400	69·5/22·0 = 3·14	30,400
5984	76·6/37·3 = 2·05	10,850	68·2/29·0 = 2·35	11,800
Mixed plasma	57/18·6 = 3·07	37,200	52·8/14·7 = 3·7	40,340
6411	67·4/26·2 = 2·58	26,000	55·4/21·2 = 2·62	26,600

* Percentage antitoxin left/percentage protein left = ratio.

TABLE X.—*Showing the Actual Amounts of Protein Digested in 7 Days at 39° C.*

Horse.	Original protein g./100 ml.	G. of protein digested after 7 days.	Percentage of original protein digested.
6193	10·80	8·05	74·4
6351	8·24	7·14	86·5
6308	11·25	8·55	76·0
5984	9·60	6·82	71·0
Mixed plasma	9·25	7·89	85·0
6411	9·07	7·15	78·7
6509 (Normal horse)	7·15	6·80	95·0

From these and many other experiments not recorded in detail the impression was gained that in plasma from certain horses there was present a protein, or proteins, which were very resistant to peptic digestion under conditions both of pH and temperature which were necessary if large losses of antitoxin were to be avoided during the digestion. At pH values below 4 or temperatures higher than 39° C. the increased digestion was associated with a marked loss of antitoxin. Generally, it was found that plasma obtained from horses which had responded well and rapidly to immunization showed good digestion, with a marked increase in antitoxic purity (about four times) and an antitoxic loss of about 25–30 per cent. Where the immunization had been slow or the response poor, the plasma proved to be far less satisfactory for this digestion process.

The Formation of a Precipitate between the Pepsin and Protein at pH 4.

When the digestions were carried out at about pH 4 it was noticed that after the addition of pepsin a precipitate formed. This was the case when the protein solution was plasma, serum or pseudoglobulin. Some observations made on this point suggest an explanation of the need for a high pepsin-protein ratio when digestions are carried out at a pH of 4.

An antitoxic pseudoglobulin solution containing 3 per cent. of protein was adjusted to pH 4·2, divided into portions, and varying amounts of pepsin added. These amounts ranged from a pepsin-protein ratio of 1–9·25 to 1–0·578. After adding the pepsin all the pH values were readjusted to 4·2. Within 24 hours, at room temperature, a precipitate had formed in all the mixtures. A portion of each solution was filtered, and the filtrate examined for antitoxin and protein. In addition a portion of each filtrate was adjusted to pH 1·5, dyed fibrin added, and then incubated at 37° C. to determine the presence or absence of pepsin in the filtrates. As shown in Table XI, only two filtrates showed the presence of pepsin by this test—the one with the lowest ratio and that with the highest ratio of pepsin to protein. After standing for 48 hours at room temperature, the filtrate from the lowest ratio gave a negative result with the fibrin test. This combination of the pepsin and some protein present renders the enzyme insoluble, but it is of interest that the precipitate so formed removes none of the antitoxic activity. This precipitate does not form at lower pH values, which may explain the fact that digestions at more acid pH values appear to require smaller amounts of enzyme.

TABLE XI.—*Showing the Combination of the Pepsin with the Precipitate Formed in an Antitoxic Pseudoglobulin Solution at pH 4·2 after 24 hours at 18° C.*

Ratio pepsin/protein.	Precipitate.	Pepsin in filtrate.	Antitoxin in filtrate (per cent.).	Protein in filtrate (per cent.).
1–9·25	±	±*	100	95
1–4·62	+	—	100	92
1–2·31	+	—	100	87
1–1·16	+	—	100	82
1–0·578	+	+	100	82

* Negative at 48 hours.

The Stability of Antitoxin at Low pH Values.

During the course of this work some observations were made which showed that the damage to antitoxin at acid pH values was very much dependent on the protein concentration, and that at low protein concentration the antitoxin was much more stable. Experiments were carried out using several acids, and those recorded in Table XII are for phosphoric acid at pH 3. It will be seen that at this pH and a temperature of 39° C. the loss of antitoxin varied from 36 per cent. to 85 per cent. in 24 hours according to the protein concentration. For a 1·0 per cent. protein solution at the same pH value, no loss of antitoxin could be detected in 48 hours at room temperature. The stability of the antitoxin in dilute solution at room temperature and a pH value of 3 suggested that peptic digestion under these conditions might have some advantage over digestion at pH 4 and higher temperatures. The 1·0 per cent. protein solution which showed no antitoxin loss in 48 hours at pH 3 gave, when treated with pepsin, a loss of 15 per cent. of its antitoxin and 65 per cent. of its protein in the same period. For an already purified pseudoglobulin the increased purity is $85/35 = 2{\cdot}42$ times as a result of this form of peptic treatment in dilute solution at low pH values and room temperature. Digestion carried out in this way at low temperature has many advantages over digestion at higher pH values and temperatures.

TABLE XII.—*Showing the Variation in Antitoxin Stability at pH* 3·0 *and* 39° *C. with Protein Concentration.* (*No Pepsin Present.*)

G. of protein per 100 ml.	Destruction of antitoxin in 24 hours at 39° C. (per cent.).
0·45	36
0·90	40
1·80	52·5
3·60	85·0

Other work which was carried out with pepsin will be considered in the next paper on the heat denaturation of the proteins of antitoxic sera.

Digestion with Activated Papain.

A study of the action of papain, suitably activated, has shown that results similar to those for pepsin may be obtained. This enzyme has, however, a number of disadvantages, amongst which the main one is the difficulty of the destruction of the enzyme at the end of the digestion. For this reason only a brief account will be given of the work carried out with papain. Most of the known activators for papain were tried; thioglycollic acid gave the best results. Difficulties were encountered in the estimation of protein by the heat coagulation method owing to the fact that further extensive digestion took place during the heating in a water-bath to 100° C. This difficulty was overcome by adding hydrogen peroxide to the acetate buffer used for the protein estimations. Fig. 2 shows the effect of hydrogen peroxide in the acetate buffer on the amount of protein found where the protein solution contained

papain activated by thioglycollic acid. The presence of 0·8 per cent. or more of 100-volume peroxide in the acetate buffer prevented hydrolysis of the protein during the estimation.

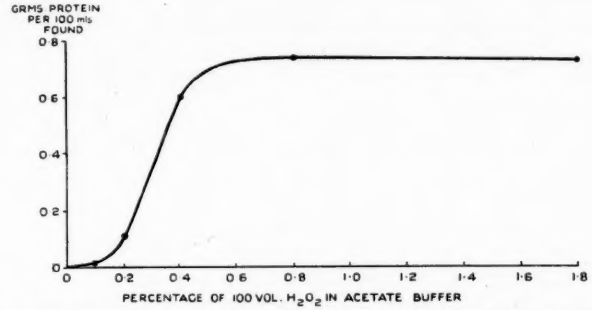

FIG. 2.—Showing the amount of peroxide required in the acetate buffer to give true protein readings, where the solutions contain papain activated with thioglycollic acid.

Antitoxic pseudoglobulin solution (500 ml.) was diluted with an equal volume of papain solution prepared as follows: 4 g. of papain and 2·5 ml. of thioglycollic acid were made to 450 ml. with water, and after standing for 30 minutes, filtered and the filtrate diluted to 500 ml. The digest solution was adjusted to pH 4·0 with solid citric acid, and heated in a water-bath at 50° C. Table XIII shows the course of this digestion with papain.

TABLE XIII.—*Showing the Digestion of Antitoxic Pseudoglobulin by Activated Papain at pH 4·0 and 50° C.*

Time at 50° C.	Percentage antitoxin left (per cent.).	Percentage protein left (per cent.).	Ratio (R. value).	Antitoxic units per g. protein.
0 minutes	100	100	1·00	10,000
15 ,,	87	67	1·30	13,000
30 ,,	79	38·5	2·05	20,500
45 ,,	72	34	2·12	21,200
60 ,,	67	30·5	2·20	22,000
90 ,,	61	27	2·26	22,600
120 ,,	56	24	2·33	23,300
180 ,,	50	19·5	2·56	25,600
240 ,,	45	17·5	2·57	25,700
300 ,,	40	16	2·50	25,000
360 ,,	37	14	2·64	26,400

From this table it will be seen that digestion with papain proceeds with greater speed than was the case with pepsin, and that in 30 minutes about 62 per cent. of the protein had been digested for a loss of 21 per cent. of the antitoxin. These figures are almost identical for this sample of antitoxin with

those obtained with pepsin, and from this point of view the action of the two enzymes can be considered as the same.

A further point of interest in connection with this experiment is the rate at which antitoxin is destroyed for each g. of protein digested. The figures calculated for the papain digestion are shown in Table XIV. Until 9 g. of protein had been digested the antitoxin loss took place at a constant rate of 4000 units per g. of protein digested, and thereafter the rate rapidly increased, being 18,000 units per g. from the 11th to the 13th g. These figures have been calculated for the original antitoxic globulin solution, which contained a total of 14·8 g. of protein per 100 ml.

Similar observations have been made for pepsin digestions, but plasma from each horse shows individual behaviour. In some cases the initial " rate loss " may be low and in others very high. Some antitoxic plasmas show a constant " rate loss " over the early part of the digestion similar to that shown in Table XIV, and others a steadily increasing " rate loss " throughout the digestion.

TABLE XIV.—*Showing the Destruction of Antitoxin during Digestion with Papain in Terms of " Units per g. of Protein per g. of Protein Digested ".*

Protein digested in g.	Rate of loss. Units per g. protein.
1st g.	4,000 units
2nd g.	4,000 ,,
3rd g.	4,000 ,,
4th g.	4,000 ,,
5th g.	4,000 ,,
6th g.	4,000 ,,
7th g.	4,000 ,,
8th g.	4,000 ,,
9th g.	4,000 ,,
10th g.	9,000 ,,
11th g.	18,000 ,,
12th g.	18,000 ,,
13th g.	18,000 ,,

Total protein available 14·8 g./100 ml.

Digestion with Trypsin.

The stability of antitoxin to trypsin depends very much on the conditions under which the digestion is carried out. Where low concentrations of trypsin are used digestion takes place very slowly, and any attempt to increase the rate of digestion by the use of large amounts of trypsin leads to a destruction of antitoxin which is approximately proportional to the amount of protein digested. By taking advantage of the fact that trypsin does not readily hydrolyse undenatured protein, it has been found possible to devise a method for its use. Attention has been directed to the complex nature of the antitoxic globulin molecule (Pope, 1938), and it has been shown that one portion of the molecule is non-antitoxic and more easily denatured than the other portion which carries the antitoxic activity. By suitable heat treatment in acid

solution, this non-antitoxic portion can be denatured and so rendered available for attack by low concentration of trypsin. There are, also, other methods by which it is possible to use trypsin, but these will be dealt with in the next paper.

The other two enzymes mentioned do not cause extensive true hydrolysis of the proteins. They also will be considered more fully in the next paper.

DISCUSSION.

In this paper it has not been possible to present more than a few of the very large number of experiments which have been carried out on the action of proteolytic enzymes on antitoxins. An attempt has been made to show some of the conditions under which these enzymes may be made to digest non-antitoxic protein and so increase the purity of the antitoxin. Observations made on other antitoxins have confirmed in general those reported in this paper for diphtheria antitoxin. The observations recorded here relate only to *the true digestion of protein as defined by its conversion into a form no longer coagulable by heat at the isoelectric point of the protein* (*in the presence of suitable electrolyte*).

In the first experiments with pepsin it was shown that for a temperature of 35°–40° C. the destruction of antitoxin at pH values of 3 or less was very marked, while at temperatures of 18°–20° C., although the antitoxin was fairly stable at pH 3, lower values than this also caused marked destruction of antitoxin. Additional evidence has been given that most of this inactivation of antitoxin is due to the effect of acid alone, but the destruction due to this cause is dependent on the protein concentration and is less in dilute solutions. However, with the low ratio of pepsin to protein used in the first experiments, there was little evidence of differential action ; the destruction of antitoxin and the digestion of protein took place to about the same degree. On increasing the pepsin-protein ratio some evidence was obtained that protein could be digested without simultaneous loss of antitoxin. An explanation of the need for a high pepsin-protein ratio has been given on the basis of precipitate formation at values of about pH 4 ; it was shown that the precipitate so formed removed all the pepsin activity from solution. In practice it has been found that little benefit is derived from a pepsin-protein ratio in excess of 1–10. An examination of various makes of pepsin has shown little difference in their action, although the most active material (B.P. 1/10,000) was slightly better. This result is not very surprising, since equal weights of the pepsins were used irrespective of their reputed activities.

One of the major difficulties encountered in the work with pepsin was that after an initial hydrolysis at a moderate rate, further digestion proceeded very slowly ; no means of increasing the digestion-rate without excessive antitoxin destruction could be found. Contrary to expectations, removal of digestion products by dialysis did not alter the rate, nor did precipitation of the undigested protein by ammonium sulphate give a product that was digested more rapidly by fresh pepsin.

The impression was gained that antitoxic plasmas contain varying amounts of protein which is only hydrolysed by pepsin with great difficulty under conditions which conserve a reasonable degree of antitoxic activity. Since all

samples of normal horse-plasma studied showed a fairly rapid digestion of about 95 per cent. of the total protein under similar conditions, it seems fair to draw the conclusion that changes arise during immunization which result in alteration of the digestibility of the serum proteins.

The results obtained from a study of the plasma from individual horses show that antitoxin stability and protein digestion varies greatly, even though all the conditions of the peptic digestion are made as nearly identical as possible. Some plasma samples show marked antitoxic loss for the digestion of the first portions of protein, while others show an antitoxin loss which is constant in terms of " units per g. protein loss, per g. of protein digested " until a very large amount of the protein has been digested.

As a result of this work, the conclusion was reached that methods involving true digestion of non-antitoxic protein in antitoxic plasma were insufficient in themselves as a method for routine purification of antitoxins. This conclusion has since been supported by the claims of U.S. Patent 2123198, which deals with a further removal of non-antitoxic proteins from digest products by adsorption methods.

Another point of practical importance which has not been considered in the experimental section of this paper arises from the fact that the true digestion of non-antitoxic protein leads to the production of proteoses. These substances are somewhat easily precipitated with the antitoxic protein if an attempt is made to isolate the antitoxin from the digestion mixture with ammonium sulphate. Their presence in the final product reduces the real increase in purity, since they must be regarded as contaminating nitrogenous substances.

In the previous paper (Pope, 1938) I have reported the fact that antitoxin altered by enzyme action shows many differences in chemical behaviour, and it is of interest here to redirect attention to the Imray Patent (1902), where it was stated that the antitoxic material, after enzyme action, was sterilized by the addition of 10 per cent. phenol. The phenol was subsequently extracted with ether. Such a concentration of phenol would have been expected to denaturate the protein and to destroy the antitoxin from what is known of the action of phenol on ordinary antitoxin. However, on repeating the directions given in the Imray patent, it was found that the enzyme-altered antitoxin survived this phenol treatment with practically no antitoxin loss.

SUMMARY.

In this paper results are given showing the conditions under which certain proteolytic enzymes cause true digestion of the proteins present in antitoxic sera. The destruction of antitoxin which takes place as a result of digestion has also been measured, and conditions determined which allow a removal of protein by digestion without a corresponding destruction of antitoxin.

The following factors are important when the enzyme used is pepsin :

(1) At temperatures of the order of 20° C., the digestion may be carried out at an acidity as low as pH 3. For higher temperatures, such as 35°–40° C., values lower than about pH 4 lead to marked antitoxin loss.

(2) In acid solutions, the protein concentration plays a large part in determining the stability of the antitoxin. Stability is greatest when the protein concentration is low.

(3) When the digestion is carried out at reactions of the order of pH 4, it is necessary to employ a high ratio of pepsin to protein because of the precipitate which forms and carries the enzyme out of solution. At lower pH values this precipitation between enzyme and substrate does not occur, and hence lower ratios of pepsin to protein may be used.

(4) Serum obtained from individual horses shows that digestibility of the non-antitoxic proteins is not a simple property; changes in the serum protein during immunization result in a very variable degree of digestion, and unpredictable antitoxin losses. Generally, serum from horses which have immunized rapidly to high titre give the best results with a simple digestion method.

(5) The production of proteoses as a result of digestion makes final isolation of the antitoxin by salting-out methods impracticable owing to co-precipitation of these substances with the antitoxin.

The other enzymes studied, trypsin and activated papain, also produce true digestion, but they also are subject to similar limits. Under no circumstances have we been able to digest as much as 90 per cent. of the original protein without prohibitive loss of antitoxin.

After the antitoxin molecule has been altered by enzyme action it shows many different properties, amongst which is a greatly enhanced stability towards phenol.

The conclusion reached from this work on true digestion by means of enzymes is that the method offers relatively little advantage over salting methods unless it is followed by the use of adsorption procedures for the further removal of inert proteins.

My thanks are due to my colleague Mr. A. T. Glenny for samples of plasma at various stages during immunizations, and for information regarding the quality of the toxoids employed.

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CULTIVATION OF THE VIRUS OF AUJESZKY'S DISEASE ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING EGG.

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DURING the course of an investigation into the existence of antibodies against the virus of Aujeszky's disease in the blood-sera of normal pigs (Glover, 1938), a series of experiments was undertaken on the immunization of laboratory animals against this virus. The results were in conformity with those of other workers on this disease and were most discouraging, since they gave no indication that any of the methods which are in current use for immunizing animals against virus diseases were of any real value for this infection.

With a view to obtaining further information on active immunization against Aujeszky's disease, it was decided to attempt the propagation of the virus on the developing chorio-allantoic membrane, in the hope that by this means a strain of modified virulence might be secured.

There is little reference in the literature to the artificial cultivation of this virus. Traub (1933) completed a series of experiments in which the Hungarian strain of Aujeszky's disease was mixed with minced rabbit testes and subsequently suspended in Tyrode's solution and rabbit serum. Preliminary titrations of the virulent rabbit brain used for the initiation of the cultures showed an M.L.D. of 2·5 mg. for mice and of 1·0 mg. for guinea-pigs. The strain was carried to the 32nd passage and was then abandoned. The amount of virus in different subcultures varied, but it may be noted that the tissues at the 25th passage had the remarkably high titre of 1 : 1,000,000.

More recently Mesrobeanu (1938) has reported that the virus can be cultivated on developing eggs by the method of Woodruff and Goodpasture (1931), where it produced a recognizable thickening of the chorio-allantoic membrane. After the 48th hour scattered greyish-white foci appeared which later became confluent, forming a uniform nap. Sections of affected membranes showed, in the early stages, a thickening and proliferation of the ectodermal cells, and later an involvement of the mesodermal and endodermal structures. The early stages were inflammatory in nature; later the cells underwent desquamation and degeneration. The nucleus was first attacked, showing commencing disintegration with oxychromic changes, but eventually the whole of the cell underwent disruption (death of the embryos is not mentioned). Six passages were effected without any evidence of alteration in the virulence of the strain.

More recently Badenski and Bruckner (1938), using the technique of Burnet, succeeded in obtaining lesions on the chorio-allantoic membrane of the hen's egg. The cultures were initiated with a suspension of infective rabbit brain. The virus, however, gradually diminished in amount with successive passages, and eventually disappeared at the 7th transference.

Cultivation of the Virus.

The Hungarian strain of the virus has been used throughout the investigation. The first passage was initiated from a suspension of virulent rabbit brain, which had an M.L.D. of 2·0 mg. for rabbits and of 5·0 mg. for guinea-pigs. The cerebral tissue was finely triturated, diluted with broth-saline to make a

1–100 suspension, and lightly centrifuged for 10 minutes. Six eggs were then inoculated by the method of Burnet (1936*b*) with 0·2 c.c. of the suspension; two were opened at the 24th hour, two at the 48th, and the others at the 3rd and 4th days respectively. At the 48th hour the membrane was slightly more opaque than the normal and showed a few whitish nodules, which were easily visible with a hand-lens. At the 3rd day macroscopic centres were apparent on both eggs, and there was a definite hæmorrhagic reaction of the whole membrane. The eggs opened on the 4th day showed no progressive changes. In each instance the embryo was dead, and the chorio-allantoic membrane was clearly undergoing autolysis. A portion of the 2-day-old membrane was ground up and suspended in broth-saline. One part was used for the inoculation of a second series of eggs, while the other was injected into a rabbit (dose, 20 mg.) and a guinea-pig (dose, 50 mg.). The rabbit was found dead at the 48th hour, while the guinea-pig died at the 3rd day. Both showed lesions typical of Aujeszky's disease.

At the moment the strain has been carried serially through 55 generations on the egg. After each passage the virus has been tested on guinea-pigs, while from time to time rabbits and mice have also been injected. The results are given in Table I, which shows the reactions at each interval of 5 transferences on the egg.

TABLE I.

Number of passages on egg.	Guinea-pig 50 mg.	Rabbit 10 mg.	Mouse 100 mg.
Original	3 D	2 D	2 D
5	4 D	—	—
10	3 D	3 D	2 D
15	3 D	—	—
20	—	5 D	—
25	5 D	—	—
30	5 D	5 D	4 D
35	5 D	—	—
40	5 D	—	—
45	6 D	—	—
49	6 D	5 D	6 D
55	6 D	—	—

D = days to death. — = not done.

Membranes in the earlier part of the series produced lesions in inoculated animals which were in no way dissimilar from those occurring with infective mammalian tissues. An intense pruritus invariably developed at or near the point of inoculation at from 6 to 24 hours before death. At autopsy there was a more or less extensive thickening of the subcutaneous tissues, which were bathed in a blood-stained serous fluid. Clear-cut hæmorrhagic areas were always present in the lungs of rabbits, and usually of guinea-pigs.

Between the 20th and 25th passage, however, a change was noted. The pruritus was less severe and eventually completely disappeared, and the local lesions were much diminished in intensity. From the 28th passage onwards

there has usually been a striking absence of a local reaction. Inoculated animals have died fairly suddenly without showing any very definite symptoms. At post-mortem examination a slight hæmorrhagic reaction has been noted in the regional lymph-glands, and a patchy congestion of the lungs. At one time it was feared that Aujeszky's virus had died out and that perhaps some other agent infective for guinea-pigs had been introduced, but serum-virus neutralization experiments showed quite clearly that Aujeszky's virus was till present in the egg.

One experiment was performed with virus in the 26th egg-passage to determine the infectivity of the organs of an inoculated guinea-pig. The animal succumbed 3 days after receiving 50 mg. Suspensions were prepared from the local lesion and from the lung, spleen and kidney; they were injected into normal guinea-pigs. All the animals died and showed lesions typical of Aujeszky's disease. The heart's blood in a dose of 0·25 c.c. failed to set up infection.

Infection of the Embryo.

As mentioned above, the embryo has usually died at the 3rd day. This applies more particularly to the earlier passages, since from about the 30th in the series the fœtal chick sometimes showed feeble movements at the 3rd day and occasionally survived up to the 4th day. Nevertheless, both living and dead embryos showed a well-marked hæmorrhagic reaction of the skin, which was most apparent in those areas which were in close proximity to the infected membrane.

Death of the embryo has been observed in other virus-infections of the egg. For example, Burnet and Galloway have described the phenomenon in louping-ill (Burnet, 1936*b*, 1936*c*), and it has also been observed in influenza (Burnet, 1936*b*), and fowl plague and Newcastle disease (Burnet and Ferry, 1934). Burnet has noted that in the last-mentioned condition the most characteristic result of infection is a development of hæmorrhages in the feather follicles, distributed mainly along the wings and on the margin of the ventral surface of the body. The gross changes occurring in embryos which have succumbed as a result of infection with the virus of Aujeszky's disease would seem to resemble those induced by the virus of Newcastle disease.

The infectivity of the embryo was tested on two occasions, viz. at the 12th and 29th passages respectively. The chorio-allantoic membrane was removed and the embryo chick carefully lifted out, care being taken to avoid contamination from the edges of the cut membrane. The fœtus was washed in several changes of saline, and the selected tissues were then excised with precautions to avoid extraneous contamination with virus. Suspensions were made in broth and were inoculated subcutaneously into guinea-pigs. In the first experiment the animals were inoculated with liver, skin, brain and eye respectively. All died with the exception of those inoculated with the liver preparation. On the second occasion (29th passage) guinea-pigs were inoculated with skin, liver, lung and brain. Those receiving skin, lung and brain died, but again the liver proved to be free from detectable virus. The fact that the liver appeared to escape infection was interesting, and suggested either that the hepatic cells were not susceptible to the virus, or that the organ excreted some inhibitory substance.

Further experiments, in which the livers from normal chick embryos were mixed with low amounts of virus, and inoculated into guinea-pigs after varying periods of contact, failed to reveal any inhibitory action on the part of the liver, and it must therefore be concluded that in all probability the hepatic cells, for some reason as yet undetermined, are not very susceptible to the virus.

Histology of the Infected Membranes.

Up to the present all viruses which have been cultivated on egg-membranes appear to induce reactions varying in extent in the ectodermal tissues. The virus of Aujeszky's disease, on the other hand, seems to behave in a rather different fashion. It is true that a certain degree of reaction of the ectoderm is seen, but it is definitely minimal in extent, and the main changes take place in the mesodermal layer, the cells of which seem to be peculiarly sensitive to the virus. Twenty-four hours after the inoculation of the egg there is a slight focal proliferation of the ectodermal cells, which probably represents the points of contact of the virus particles. The mesoderm is considerably thickened, œdematous, and shows commencing infiltration with acidophilic leucocytes. At the 48th hour the proliferation of the ectodermal cells has ceased, but in those sites where some increase in thickness has occurred the outer layer of cells has commenced to undergo necrosis. The infiltration with leucocytes in the mesoderm is not much increased, but the mesodermal cells show great activity, as evidenced by the presence of numbers of stellate cells with large nuclei. Some of these appear to arise from the neighbourhood of the blood-vessels, but the majority of these cells show no immediate association with the capillaries. By the 3rd day the mesodermal reaction has reached its highest level. At this stage the nuclei of the cells commence to break up, and a degenerative process of the type of " chromatotaxis " can be clearly recognized. This stage, however, rapidly passes on to one in which there is complete necrosis of the whole cell architecture.

Mutation of the Virus in the Developing Egg.

The effect of repeated passage of the virus on the chorio-allantoic membrane has been studied from both a quantitative and qualitative point of view. Changes in the concentration of virus in the egg have been determined by comparing the minimum lethal doses of similar amounts of egg-membrane virus and virulent rabbit brain for the guinea-pig and mouse respectively, while modifications in the length of period elapsing between inoculation and death and the character of the resulting lesions have been adopted as the criteria for the determination of alterations in the character of the virus.

TABLE II.

	Guinea-pigs.				Rabbits.			Mice.			
	50 mg.	20 mg.	5 mg.	1 mg.	10 mg.	1 mg.	0·2 mg.	100 mg.	20 mg.	5 mg.	2·5 mg.
Original brain.	D	D	D	S	D	D	S	D	D	S	—
21st passage .	D	D	D	S	—	—	—	D	D	D	—
36th passage .	D	D	D	D	D	S	S	D	D	D	D

D = died. S = survived. — = not done.

An examination of Table II, giving the M.L.D.'s for mice and guinea-pigs of original rabbit brain and 21st and 36th passages on egg respectively, shows that there has been no loss in infectivity. For example, the M.L.D.'s of the original material for guinea-pigs and mice were 5 mg. and 7·5 mg. respectively. At the 21st passage 5 mg. was still the lowest dose infective for the guinea-pig, but the same dose now killed the mouse, while after 36 transferences the M.L.D. had been reduced to less than 2·5 mg. The infectivity of the membrane was therefore fully maintained, or even slightly augmented. On the other hand, there was a steady increase in the duration of the experimental disease. Thus, guinea-pigs inoculated with 50 mg. of the original cerebral tissue succumbed after 48 hours, while those animals receiving a similar weight of the chorio-allantoic membrane in the 21st passage died in 5 days, and in the 36th passage in 6 days. The difference in mice was even more striking, death occurring in from 2 to 4 days after inoculation with the original material, and in from 7 to 10 days at the 36th passage. Of itself, an increase in the duration of the experimental disease does not necessarily imply a modification of the virus in the sense that it is undergoing attenuation, but when taken in conjunction with the further changes in character which have already been mentioned, viz. an abrupt disappearance of pruritus and almost complete absence of a local lesion and a general alleviation of the symptoms, it seems legitimate to infer that the virus is undergoing modification. It would be unwise, however, to draw any final conclusion on this point, since Aujeszky's disease virus can become attenuated for one species without losing any of its pathogenicity for another. Thus Shope (1933) has shown that pseudo-rabies virus passaged intracerebrally through the guinea-pig becomes apathogenic for this species when introduced by the subcutaneous route, although the strain still appears to retain its full powers of infectivity for the rabbit.

Serum-Virus Mixtures.

The experiments of Burnet and other workers on influenza, louping-ill, Newcastle disease, etc., have demonstrated that the neutralizing action of immune sera can be assayed on the egg membrane. Burnet has used the " pock-counting " method, in which fixed amounts of virus are added to decreasing amounts of serum, and the mixtures, after a suitable period of incubation, are introduced to the chorio-allantoic membrane. The neutralizing action of the serum is evaluated by detecting the reduction in countable pocks as compared with mixtures of virus and normal serum. Experiments with Aujeszky's disease virus have proved particularly interesting. Potent antiviral sera can be readily obtained which neutralize the virus *in vitro*, but they are apparently without effect on the developing egg. Table III shows the effect of mixing diminishing amounts of an anti-serum prepared in the pig (Glover, 1938) with fixed amounts of virulent rabbit brain and infective egg-membrane respectively, the mixtures being inoculated into guinea-pigs after contact at room temperature for 4 hours. In each case approximately 0·02 c.c. of serum completely inactivated about 20 guinea-pig M.L.D. of virus. At the same time 0·1 c.c. of each suspension was inoculated into an egg. When opened at the 3rd day no differences could be detected, since in every instance the membrane

TABLE III.

Virus.	Serum.	Time.	Guinea-pig test of mixtures.	Guinea-pig test of egg-membranes.
Rab. brain, 20 mg.	I.S. 0·1 c.c.	½ hour at 22° C.	S	D
,, ,,	,, 0·05 c.c.	,, ,,	S	D
,, ,,	,, 0·02 c.c.	,, ,,	S	D
,, ,,	,, 0·01 c.c.	,, ,,	D	D
,, ,,	N.S. 0·1 c.c.	,, ,,	D	D
Rab. brain, 100 mg.	I.S. 0·2 c.c.	½ hour at 22° C.	S	D
,, ,,	,, 0·1 c.c.	,, ,,	S	—
,, ,,	,, 0·05 c.c.	,, ,,	D	—
,, ,,	,, 0·02 c.c.	,, ,,	D	D
,, ,,	N.S. 0·2 c.c.	,, ,,	D	D
Egg membrane, 28th 100 mg.	I.S. 0·2 c.c.	½ hour at 22° C.	S	D
,, ,,	,, 0·1 c.c.	,, ,,	S	D
,, ,,	,, 0·05 c.c.	,, ,,	S	D
,, ,,	,, 0·02 c.c.	,, ,,	D	D
,, ,,	N.S. 0·2 c.c.	,, ,,	D	D

I.S. = immune sera ; N.S. = normal serum ; — = not done ; S = survived ; D = died.

showed a uniform diffuse necrosis, even in the case of mixtures containing the largest amount of serum. Guinea-pigs inoculated with these membranes all died of Aujeszky's disease. The experiment has been repeated on two other occasions with similar results. The addition of complement to the mixtures prior to their introduction to the eggs failed to prevent this dissociation.

These findings are in marked contrast to those obtained by Burnet and other workers with different viruses cultivated on the egg. In general, it has been found that membranes inoculated with serum-virus mixtures in which the components have been in contact for a short time prior to their introduction to the egg, have shown an appreciable reduction in the number of " pocks " as compared with those inoculated with virus plus normal serum. In the case of influenza the results are even more striking, since immune serum introduced to the egg *after* the application of the virus is still capable of affording some degree of protection (Burnet, 1936*a*). Since mixtures of Aujeszky disease virus and immune serum appear to behave in a different manner, a further experiment was performed in which anti-serum was applied to the membrane *before* the virus to determine whether its entry into the cells could be prevented.

Four eleven-day-old eggs were opened and artificial sacs were formed in the usual manner, after which 0·2 c.c. of Aujeszky immune serum was dropped on to the membrane ; at the same time 4 more eggs received 0·2 c.c. of normal serum. The eggs were closed and returned to the incubator for varying periods. One egg from each batch was withdrawn at intervals of 15 minutes, 30 minutes, 1½ hours and 2½ hours respectively, the cover-slip removed, and 0·2 c.c. of a suspension of Aujeszky disease virus containing 20 guinea-pig M.L.D.'s was applied to the membrane. The eggs were then incubated for a period of 3 days, the membranes removed and inoculated into guinea-pigs.

The results showed that up to and including half-an-hour the anti-serum failed to prevent infection of the cells of the chorio-allantoic membrane, since the guinea-pigs died from Aujeszky's disease within the same period as controls. When the anti-serum had acted for 1½ to 2 hours on the membranes they proved to be non-infective.

DISCUSSION.

The data given in this paper indicate that the Hungarian strain of Aujeszky disease virus can be cultivated on the chorio-allantoic membrane of the developing egg without difficulty. The observations of Mesrobeanu (1938) on the suitability of this method for the propagation of the virus are thus confirmed. Attention may be directed to the unsuccessful efforts of Badensky and Bruckner (1938), who were unable to establish the virus on the membrane. In their article they suggest that the maximum activity of the virus occurs about the 20th hour and that, thenceforward, there is a sudden decline in virulence. They postulate that in order to obtain serial cultures it may be necessary to passage the virus every 24 hours. The experiments recorded in this paper afford no evidence that such frequent passages are necessary, since no difficulty was experienced in initiating fresh cultures at the 3rd or even 4th day. It may be suggested that the early disappearance of the virus (7th passage) in the experiments of Badensky and Bruckner may have been due to the employment

of a very small inoculum. They mention that they used 0·04 c.c. at each passage,* whereas in the experiments recorded in this paper no less than 0·2 c.c. of about a 1–100 dilution was used to start each fresh generation.

Mesrobeanu (1938) failed to obtain any evidence of attenuation in the virulence of Aujeszky disease virus by passage on the egg, but her cultures were not taken beyond the 6th generation. In these experiments no evidence of mutation was induced before the 20th generation, but thenceforward a recognizable alteration occurred. The original material produced the classical symptoms in rabbits, guinea-pigs, etc., viz. an intense pruritus at the point of injection and death within 2 to 3 days ; at autopsy well-marked changes were seen at the local site and in the lungs. The adaptation of the virus to the egg membrane resulted in a loss in the capacity to induce the local pruritus, an increase in the period of incubation, and finally, a marked diminution in the severity of the pulmonary lesions. It must be admitted, however, that egg-virus, although modified, is still highly lethal for rabbits, guinea-pigs and mice, and cannot yet be used with safety as an immunizing agent. Possibly a more prolonged series of passages on the egg membrane might produce an attenuated strain which could be used for this purpose.

The application of mixtures of Aujeszky disease virus and immune serum to the egg membrane is of interest. It has been established with other viruses that properly balanced mixtures of virus and immune serum, which are innocuous when introduced into susceptible animals, may be dissociated to some extent when applied to the chorio-allantoic membrane. Nevertheless, the liberation of active virus is only partial, since there is usually an appreciable reduction of the numbers of countable " pocks " as compared with controls. With Aujeszky's disease it is difficult to obtain isolated lesions on the membrane owing to the rapid spread of the virus, but the available evidence suggests that neutral serum-virus mixtures undergo considerable dissociation in the egg, since they produce lesions on the chorio-allantoic membrane which are as marked as those induced by the mixtures of normal serum and virus. Membranes inoculated with serum-virus mixtures apparently contain as much virus after incubation as those inoculated with an equal dose of virus alone.

CONCLUSIONS.

(1) The virus of Aujeszky's disease has been cultivated on the chorio-allantoic membrane of the developing egg to the 55th generation.

(2) The strain has become modified in its effect on laboratory animals. The period of incubation has been lengthened, local pruritus is no longer produced, and the characteristic pulmonary lesions are much diminished in intensity ; nevertheless, the virus is still as lethal as infective rabbit brain.

(3) Mixtures of immune serum and virus which are innocuous to experimental animals undergo dissociation on the egg membrane. Protection of the chorio-allantoic cells can be secured by the application of immune serum not less than half-an-hour *before* the virus.

* Dilution not stated.

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THE IMMUNOLOGICAL RELATIONSHIP OF THE VIRUS OF SPONTANEOUS COWPOX TO VACCINIA VIRUS.

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THE observations of Jenner (1798) indicated the close immunological relationship between the virus of cowpox and that of smallpox, but it is evident from the publications of Crookshank (1889), Cory (1898) and Copeman (1899) that, of the so-called " vaccinia " strains in current use in various parts of the world towards the end of last century, many had been derived by passing human smallpox through animals. The more recent literature on the transformation of variola to vaccinia has recently been reviewed and discussed in relation to his own observations by Horgan (1938).

Although the terms " vaccinia " and " cowpox " are frequently used synonymously, it seems doubtful whether the viruses concerned should be regarded as identical, yet in much of the earlier work on the protective power of " vaccinia " against the virus of smallpox in man or monkeys (Copeman) such identity is assumed. The more recent observations of Green (1908), Reece (1921), Van Heelsbergen (1922), Frenkel (1930), Glover (1937) and others have shown that the virus of spontaneous cowpox and laboratory strains of vaccinia produce effective immunity against each other in animals.

While there doubtless exists a close relationship between these viruses, the examination of convalescent sera from three men who had contracted infection from the spontaneous disease in cattle (Davies, Janes and Downie, 1938)

suggested that the virus isolated from one of the men was not identical with a strain of vaccinia in current use. A comparative study of the lesions produced experimentally by three strains derived from the natural disease in the cow and three strains of vaccinia also indicated differences which have been described elsewhere (Downie, 1939). The methods which have recently been developed by Craigie (1932) have provided a means whereby a more accurate knowledge of the antigens of vaccinia virus and the antibodies in antivaccinial serum has been gained (Craigie and Wishart, 1934*a* and 1936 ; Parker and Rivers, 1935 ; Salaman, 1937). By utilizing the methods developed by these workers for the preparation of relatively pure suspensions of virus and the carrying out of serological tests, an attempt has been made to determine the immunological relationship between two strains of vaccinia virus and two strains of the virus of spontaneous cowpox. The results of these investigations are recorded in this paper.

MATERIAL AND METHODS.

Strains of Virus.

Cowpox virus, strain B, was isolated from the lesions on the hands of a cowman on a farm in Sussex in 1937, and had been passed by pad inoculation through two guinea-pigs, and thereafter by intradermal inoculation or by scarification in the skin of rabbits.

Cowpox virus, strain G, was isolated in 1937 from scabs derived from typical lesions on a cow by Mr. R. E. Glover, of the National Institute for Medical Research, Millhill. It was passed intradermally through 3 rabbits, then by intratesticular inoculation through two rabbits, and thereafter by intradermal or cutaneous inoculation on these animals.

Vaccinia virus, strain S.—This was the stock Lister Institute strain which had been passed repeatedly by cutaneous inoculation in rabbits, and was used by Dr. M. H. Salaman in his work on the combining properties of vaccinia virus with the antibodies demonstrable in antivaccinial serum (Salaman, 1937). This strain has been in use at the Lister Institute for many years, but it is uncertain whether it was originally obtained from spontaneous cowpox or from variola in man.

Vaccinia virus, strain A, was derived by Dr. C. R. Amies from smallpox material obtained in 1932, and had been repeatedly passed in rabbits by cutaneous inoculation.

An account of the lesions produced by these four strains of virus on the chorio-allantoic membranes of developing hens' eggs, in guinea-pigs and in rabbits has been reported elsewhere. In the experiments recorded below all immunity tests were made in rabbits. In some of the earlier experiments the supernatant fluid from infected egg-membranes or from the ground-up lesions which had resulted from intradermal injection in the rabbit was the infective material, but in all later experiments elementary body suspension prepared from cutaneous infection in rabbits has been used except in one instance, when a similar suspension was made from infected egg-membranes.

Preparation of Elementary Body Suspensions and Soluble Specific Substance.

The method of Craigie (1932) as modified by Salaman (1937) was used throughout. Rabbits were inoculated on the shaved and lightly scarified skin of the back with elementary body suspension and were killed and exsanguinated on the 3rd or 4th day afterwards. Elementary body suspension and crude Seitz filtrates containing the soluble specific substance were prepared according to the technique described in detail by Salaman using M/250 citric acid + disodium phosphate buffer pH 7·1 for successive washings and final suspension. It may be observed that while the titre of soluble specific substance obtained from the four strains of virus was approximately the same as indicated by complement-fixation tests with appropriate immune sera, the yield of thrice washed elementary bodies was usually greater with vaccinia virus, strain S, than with the other three strains. Examination of the suspensions under dark-ground illumination and by stained films indicated that they were relatively pure, although with the three latter strains a certain amount of ill-defined, finely granular material less refractile than the elementary bodies was frequently present in the final suspensions. The number of bacteria in the suspensions was always estimated by cultures made in blood-agar, and these showed counts varying from three to several hundred colonies from 0·1 c.c. When suspensions were not required for immediate use they were stored at a temperature of 0° to 4° C. under ether.

Preparation of Antisera.

In certain experiments the sera from rabbits which had received multiple intradermal injections of virus 2 to 4 weeks previously were used, but the sera for absorption experiments were obtained from animals which had received two to four further injections of virulent elementary body suspension. These injections were made intraperitoneally or intravenously at intervals of 7 to 14 days, and the rabbits were bled 6 to 8 days after injection. The sera were heated at 56° C. for 40 minutes and were stored without preservative. In general it has been found that good antisera are more easily obtained against the cowpox than against the vaccinia strains. Sera from rabbits which had been infected intradermally with cowpox virus two or three weeks previously showed good neutralizing power and a good titre of complement-fixing antibody whereas, with the vaccinia strains, hyperimmunization was usually necessary to obtain sera with a satisfactory antibody content.

Agglutination Tests with Elementary Body Suspensions.

The method used was that described by Craigie and Wishart (1934*a*). Dilutions of serum were made in 0·85 per cent. NaCl containing M/250 citric acid + disodium phosphate, pH 7·1. Stock suspensions of elementary bodies were diluted before use in M/250 buffer to give a turbidity comparable to that of bacterial suspensions suitable for agglutination. Equal volumes (0·3 c.c.) of serum dilutions and suspension were mixed in small tubes, which were then incubated under cover in a water-bath at 50° C. The results were read after

3 hours and again after 18 to 20 hours. Normal rabbit serum was always included for control purposes.

Complement-fixation Tests.

The complement-fixation reaction with soluble precipitable substance contained in Seitz filtrates of vaccinia skin pulp and anti-vaccinial sera has been found by Craigie and Wishart (1934*b*) to give results comparable to precipitation tests. In preliminary tests made with the Seitz filtrates prepared from the cowpox viruses used in this work, the complement-fixation reaction was found to be more sensitive and to give sharper results than the precipitation test, and in consequence has been used extensively for the titration of soluble specific substance or antibody in immune sera.

To 0·2 c.c. quantities of a suitable dilution of serum or filtrate 0·2 c.c. of guinea-pig serum diluted to contain 3 M.D.H. of complement and 0·2 c.c. of dilutions of filtrate or serum were added. The tubes were kept at room temperature for 4 hours before the addition of 0·4 c.c. of sensitized cells (equal volumes of 5 per cent. sheep cells and amboceptor containing 5 M.D.H.). The tubes were then placed in a water-bath at 37° C. for 20 to 30 minutes and afterwards at room temperature. The results were read after 1 hour, and again after the racks had been left on the bench overnight. Suitable controls of antigen and serum were included in each test. In preliminary tests the findings of Craigie and Wishart in respect of the more complete fixation obtained at 4° C. for 16 hours were confirmed, but for most purposes fixation for 4 hours at room temperature gave satisfactory results and has been used throughout.

Absorption of Sera.

In attempting to determine immunological differences between the cowpox and vaccinia strains, cross-absorption of hyperimmune sera with elementary body suspensions has been used. It has been noted by Salaman (1937) that more complete absorption of an antivaccinial serum is obtained when the absorption is carried out with three successive fractions than when a single absorption with the same dose of elementary bodies is made. For the purpose of the present work complete absorption of sera was not considered necessary, and it has been found that, with most sera, adequate absorption of homologous antibody could be obtained by using the yield of elementary bodies from one rabbit for 1·0 c.c. of immune serum. Prior to absorption of sera the washed elementary body suspension was spun out in the angle centrifuge, and the deposit taken up in 2–3 c.c. of M/250 citric acid + disodium phosphate buffer. To 1·0 c.c. of immune serum one-third of the elementary body suspension was added, and the volume made up to 3·0 c.c. with distilled water and 8·5 per cent. salt solution to give a final concentration of 0·85 per cent. The mixture was allowed to stand at room temperature for 1 hour and then placed at 0°–4° C. overnight. Next day the mixture was spun in the angle centrifuge and the clear supernatant pipetted off. The second fraction of elementary body suspension was added, and the volume made up to 4 c.c. with distilled water and 8·5 per cent. as before, and absorption allowed to proceed as in the first instance. The third absorption was similarly made and the final supernatant

of 5·0 c.c., representing a 1 in 5 dilution of the original serum, was heated at a temperature of 56° C. for 1 hour to destroy any live virus which might have been present. Control unabsorbed sera were always subjected to the same temperature as absorbed sera. The absorbed sera were tested for residual agglutinins, complement-fixing and neutralizing antibody. In certain of the earlier experiments the mixtures were placed in a water-bath at 50° C. for 2 hours during absorption, but equally good absorption seemed to occur when the mixtures were kept at 0°–4° C. overnight, and this procedure was followed in most of the experiments recorded below.

Neutralization Tests.

Equal volumes of 1/5 dilutions of absorbed or unabsorbed sera were mixed with falling 10-fold dilutions made in broth of elementary body suspension of the virus under examination. After the tubes had been kept at room temperature for 1 hour 0·2 c.c. of each mixture was injected intradermally into normal rabbits. All sera to be compared were titrated on the same animals, two rabbits being used for the injection of the same serum-virus mixtures in each test. The readings recorded in the following tables were made on the 4th or 5th day after injection.

Housing of Animals.

Owing to the limited accommodation available it was impossible to keep animals infected with the four viruses in separate animal houses. Animals infected with cowpox strain B and cowpox strain G were kept in different rooms in the same building, while the rabbits infected with the vaccinia strains were kept in one room in another building several hundred yards from the first. The experiments with the two vaccinia strains were so arranged that animals used for preparation of elementary body suspensions of the two strains were not kept in the one animal house at the same time. In certain experiments made to test resistance of immunized animals and involving the injection of different strains of virus, these animals were removed when necessary to the building used for the vaccinia work immediately before the test injections were made. Animals infected with the vaccinia strains were never kept in the building which housed the animals used for the cowpox viruses. These precautions were considered necessary in view of the ease with which vaccinia is reported to spread among rabbits kept in the same room.

EXPERIMENTAL.

Cross-immunity in Rabbits Recovered from Infection with Cowpox or Vaccinia Virus.

Many of the animals used for titration of cowpox strain B and vaccinia strain S were later tested for resistance to intradermal injection of these two strains. Table I shows the results of such tests on two rabbits which recovered from infection with vaccinia S (V113 and V267), and two which had recovered from infection with cowpox B (B34 and B44). The first three animals shown

TABLE I.—*Cross immunity Tests in Animals Recovered from Infection with Cowpox Virus, Strain B, and Vaccinia, Strain S.*

Rabbit number.	Previous infection with—	Interval between infection and test.	Tested by intradermal injection of—											
			Vaccinia, strain S.						Cowpox, strain B.					
			Dilutions of virus.											
			10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
V. 113	Vaccinia S	21 days	+	+	+	±	−	O	+	+		±	−	O
V. 267	,,	14 ,,	+	+	+	−	−	O	+	±	±	−	−	O
Normal rabbit	..	..	++	++	++	++	+	±	++	++	++	++	+	±
B. 34	Cowpox B	30 ,,	+	+	+	±	±	O	±	±	±	±	−	O
B. 44	,,	15 ,,	+	+	±	±	−	O	+	±	−	−	−	O
Normal rabbit	..	..	++	++	++	+	+	±	++	++	++	++	+	±

++ Signifies a lesion of average diameter 15 mm. or more.
+ ,, ,, ,, 7–15 mm.
± ,, a tiny papule less than 7 mm. in diameter.
− ,, complete absence of reaction.
O ,, not tested.

TABLE II.—*Cross-immunity Tests on Hyperimmunized Rabbits.*

Rabbit number.	Immunized with—	Interval between last immunizing injection and test.	Tested with—	Dilutions of virus injected intradermally.							
				10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
B. 82	Cowpox B	8 weeks	Cowpox B	+	±	−	−	−	−	..	..
			,, G	+	+	−	−	−	−	..	..
			Vaccinia S	+	+	±	−	−	−	..	..
B. 83	,, B	8 ,,	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	+	+	±	−	−	..	..
			Vaccinia S	+	+	+	+	±	−	..	..
G. 101	,, G	11 days	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	+	+	−	−	−	..	..
			Vaccinia S	+	+	+	+	−	−	..	..
G. 105	,, G	11 ,,	Cowpox B	+	−	−	−	−	−	..	..
			,, G	+	−	−	−	−	−	..	..
			Vaccinia S	+	+	−	−	−	−	..	..
139	Normal animal		Cowpox B	..	..	++	++	++	++	+	−
			,, G	..	..	++	++	++	++	±	−
			Vaccinia S	..	..	++	++	++	++	+	±
140	,, ,,		Cowpox B	..	..	++	++	++	+	−	−
			,, G	..	..	++	++	++	+	±	−
			Vaccinia S	..	..	++	++	++	+	±	−
V. 136	Vaccinia S	21 days	Cowpox B	+	+	±	−	−	−	..	..
			,, G	+	±	−	−	−	−	..	..
			Vaccinia S	+	+	±	−	−	−	..	..
196	Normal animal		Cowpox B	..	..	++	++	++	++	±	−
			,, G	..	..	++	++	+	±	−	−
			Vaccinia S	..	..	++	++	+	±	−	−
V. 273	Vaccinia A	10 ,,	Cowpox B	−	−	−	−	−	−	..	..
			Vaccinia S	++	++	+	−	−	−	..	..
			,, A	+	+	±	−	−	−	..	..
V. 276	,, A	10 ,,	Cowpox B	+	+	−	−	−	−	..	..
			Vaccinia S	++	++	+	+	−	−	..	..
			,, A	++	+	+	−	−	−	..	..
286	Normal animal		Cowpox B	..	..	++	++	++	±	−	−
			Vaccinia S	..	..	++	++	++	++	+	±
			,, A	..	..	++	++	++	+	±	−
287	,, ,,		Cowpox B	..	..	++	+	+	−	−	−
			Vaccinia S	..	..	++	++	++	++	+	−
			,, A	..	..	++	++	+	±	−	−

The signs have the same significance as in Table I.

in the table were tested 10 days before the other three. In the first test the cowpox material used for infection was the supernatant fluid from a suspension of tissue obtained from a 5-day intradermal lesion, while for the test with cowpox B in the other three animals culture virus from the 20th passage on eggs was used. The same elementary body suspension of vaccinia virus, prepared from infected rabbit-skin pulp, was used in both tests.

The results suggest that the rabbits recovered from infection with cowpox were rather more resistant to this strain than to vaccinia virus. The two groups of animals are obviously not strictly comparable, however, and it is evident that the rabbits which had recovered from infection with either of the two viruses showed considerable resistance to both.

Rabbits which had been used for the preparation of hyperimmune sera were also tested for resistance to infection with cowpox and vaccinia viruses. In these tests elementary body suspensions were used for the intradermal injections. All such suspensions were prepared from rabbit-skin lesions except in the last four animals, for which the elementary body suspension of cowpox strain B was prepared from infected egg-membranes of the 69th passage. The results of the tests on these hyperimmunized animals are shown in Table II.

The first six animals were injected on the same day with the same dilutions of elementary body suspensions; rabbits V136 and 196 were tested ou a later date, while the last four animals were tested together still later. Although there may have been a slight variation in the infective titre of the virus suspensions used in the three groups of animals, as indicated by the lesions produced in the control normal rabbits, it is obvious that hyperimmunization with living virus of any of the four strains induces a high degree of resistance to both cowpox and vaccinia.

Neutralization Tests with Convalescent Sera.

Various sera obtained from rabbits from 18 to 24 days after intradermal titration of strains cowpox B and vaccinia S were tested for neutralization against these two viruses. All sera obtained from cowpox convalescent rabbits showed good neutralization, but the sera of rabbits recovered from vaccinial infection were more variable in this respect. One weak antivaccinial serum showed some neutralizing power against vaccinia S virus, but little or none against cowpox B. The antivaccinial serum used for the experiments on Table III was collected from a rabbit 21 days after intradermal titration of strain S and showed a good neutralizing titre. The anticowpox serum was obtained 19 days after intradermal titration of cowpox strain B. The sera had been inactivated and were added, without dilution, to 10-fold dilutions of virus in preparing the mixtures for intradermal injection.

The results shown in Table III indicate that the convalescent sera from rabbits previously infected with either strain of virus neutralizes both. However, certain weak antivaccinial sera seemed to possess little or no neutralizing antibody for cowpox virus, and tests with the sera of three cowmen who had recovered from infection with cowpox virus showed considerable neutralizing antibody against this virus, but little against vaccinia virus, strain S (Davies,

TABLE III.—*Neutralization of Cowpox and Vaccinia Viruses with Rabbit Convalescent Sera.*

Sera in mixtures.	Rabbit number.	Dilutions of virus in mixtures with undiluted sera.											
		Cowpox B.						Rabbit number.	Vaccinia S.					
		10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.		10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
Anticowpox B	45	−	−	−	−	−	O	37	±	−	−	−	−	O
Antivaccinial S		−	−	−	−	−	O		+	−	−	−	−	O
Normal rabbit		++	++	++	++	+	±		++	++	++	+	+	−
Anticowpox B	46	−	−	−	−	−	O	38	±	−	−	−	−	O
Antivaccinial S		−	−	−	−	−	O		+	±	−	−	−	O
Normal rabbit		++	++	++	++	+	±		++	++	++	++	+	±

The signs have the same significance as in Table I.

TABLE IV.—*Complement-fixation Tests with the Antisera to Two Cowpox Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Absorbed with E.Bs. of strain—	Antigens.									
		Cowpox B, Seitz filtrate.				Cowpox G, Seitz filtrate.				No antigen serum controls.	
		Serum dilutions.									
		1/20.	1/40.	1/80.	1/160.	1/20.	1/40.	1/80.	1/160.	1/20.	1/40.
Anti-B	Cowpox B	++++	+++	+	−	++++	++	−	−	−	−
	,, G	++++	+++	+	−	++++	+	−	−	−	−
	nil	++++	++++	++++	++	++++	++++	+++	+	−	..
Anti-G	Cowpox B	++++	+++	−	−	++++	++	−	−	+	−
	,, G	+++	++	−	−	+++	−	−	−	−	−
	nil	++++	++++	++++	+++	++++	++++	++++	++	−	..
Normal rabbit	*nil*	±	−	..	..	−	−	..	..	..	..

E.Bs. = Elementary bodies.
++++ signifies no hæmolysis, complete fixation.
− signifies complete hæmolysis, no fixation.
+++, ++, +, ±, ∓ increasing degrees of hæmolysis.

Janes and Downie, 1938). In view of these findings and the differences observed in the histology of the lesions produced experimentally by cowpox and vaccinia strains (Downie, 1939), it seemed possible that antigenic differences between these virus strains might be brought out by cross-absorption tests with hyperimmune sera. The results of such tests are recorded in the next section.

Cross-absorption Tests with Hyperimmune Sera.

Comparison of the Two Cowpox Strains.

The two cowpox strains produced identical lesions on animals and on the chorio-allantoic membranes of hens' eggs, and infection experiments on recovered animals failed to indicate immunological differences. However, they had been isolated in different parts of the country, and cross-absorption of hyperimmune sera with elementary body suspension was carried out to determine whether there were minor serological differences. The result of the tests with these two strains will serve to indicate the value of the methods used in the further examination of the cowpox and vaccinia strains. The absorption of each of the two sera by the two viruses was carried out under the same conditions at the same time. In each instance the first and second fractions of the total absorbing dose of elementary bodies were completely agglutinated after contact with the serum overnight in the ice-chest, while the third fraction was only partially agglutinated and the supernatant fluid remained very turbid. The four samples of absorbed serum obtained by centrifugation for 1 hour in the angle centrifuge represented a dilution of 1/5 of the original sera, and were heated for 1 hour at 56° C. together with the unabsorbed sera and normal rabbit serum similarly diluted. The seven samples of serum were then used for complement-fixation, agglutination and neutralization tests. The results are shown in Tables IV, V and VI. Four rabbits were used for the neutralization tests, the seven sera being tested against each virus in duplicate ; Table VI shows the readings from one rabbit of each pair.

From the results of the complement-fixation tests it is apparent that although absorption of the sera is not complete, the reduction in titre of each serum effected by the two strains of virus is approximately the same. The same relationship holds in the agglutination tests, although in this case the absorption of antiserum to cowpox G would appear to be almost complete. This difference in the results of complement-fixation and agglutination tests with absorbed sera has been observed throughout this work, and illustrates the greater delicacy of the complement-fixation test as a measure of residual antibody in absorbed sera. It has been shown by Craigie (1932), and Craigie and Wishart (1936), that the antigens in Seitz filtrates of crude vaccinia suspensions responsible for precipitation with antivaccinial serum are also concerned with the agglutination of elementary bodies by the same serum, and their work on complement-fixation (Craigie and Wishart, 1934*b*) suggests that here also the same antigens are concerned in the reaction with immune serum. Observations made in the course of the present work with cowpox

TABLE V.—*Agglutination Tests with the Sera shown in Table IV.*

Sera.	Absorbed with E.Bs. of strain—	E.B. suspensions used. Cowpox B. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Cowpox G. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Anti-B	Cowpox B	++	+	±	−	−	−	+	+	−	−	−	−
	,, G	++	+	±	−	−	−	+	±	−	−	−	−
	nil	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	++	±
Anti-G	Cowpox B	+	−	−	−	−	−	±	−	−	−	−	−
	,, G	±	−	−	−	−	−	−	−	−	−	−	−
	nil	+++	+++	+++	+++	++	+	+++	+++	++	++	+	±
Normal rabbit	*nil*	−	−	−	..	..	..	−	−	−	..	..	..

+++ = Complete agglutination and sedimentation.
++ = Agglutination with formation of coarse granules.
+ = Formation of granules easily visible with naked eye.
± = Formation of fine granules visible with the hand lens.
− = No agglutination.

TABLE VI.—*Neutralization Tests with the Sera shown in Table IV.*

Sera in mixtures.	Sera absorbed with E.Bs. of strain—	Virus in mixture. Cowpox B. Dilutions of virus added to 1/5 dilution of sera. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Cowpox G. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}	Controls (serum alone).
Anti-B	Cowpox B	20	16	17	7	—	..	15	15	10	5	—	..	—
	,, G	20	17	19	13	—	..	19	17	14	6	—	..	—
	nil	7	6	—	—	—	..	—	—	—	—	—	..	..
Anti-G	Cowpox B	24	24	26	22	11	..	21	20	17	12	4	..	—
	,, G	26	25	24	18	9	..	21	17	14	10	7	..	—
	nil	7	5	—	—	—	..	—	—	—	—	—	..	..
Normal rabbit	*nil*	26	26	28	24	14	5	21	21	19	16	9	3	..

Figures indicate the average diameter of the lesions in millimetres.

virus and its antiserum have yielded no evidence to suggest that complement-fixation tests with Seitz filtrates and agglutination of elementary bodies are due to different antibodies in cowpox antisera.

The results of the neutralization test shown on Table VI are similar to those obtained with the other two tests. The amount of elementary body suspension used for absorption of the two sera was the same, and the more complete exhaustion of the antiserum to cowpox G with both suspensions may have been due to the smaller amount or possibly the greater avidity of the antibody in the unabsorbed serum. Nevertheless the results of the tests with the absorbed sera give no indication of antigenic difference between these two cowpox strains.

It should be noted that in carrying out the neutralization tests the serum concentration is kept constant in the mixtures with dilutions of virus, which is the reverse of the procedure used in the agglutination and complement tests, so that exact parallelism in the results of neutralization and the other two tests with absorbed serum is hardly to be expected. There is some evidence from the work of Parker and Rivers (1936) and Salaman (1937) that neutralization of virus and precipitation with Seitz filtrates of vaccinia may not be due to the same antibody in antivaccinial serum. Nevertheless the active elementary bodies used for absorption of immune sera in the present experiments presumably contain all the antigens concerned in the reactions, and a similar reduction in titre of antibody in absorbed sera has been shown by the three methods of testing.

Comparison of the Two Vaccinia Strains.

In view of the history of the two vaccinia strains, one of which (strain A) was known to have been derived from human smallpox material, they were compared by the method of serum absorption as used for the cowpox strains. Strain A had not recently been passed so frequently by cutaneous inoculation of rabbits, and in consequence the amount of elementary body suspension recovered from each rabbit was less than in the case of strain S. The absorption of the two antisera was carried out in the same way as with the cowpox antisera, but unfortunately part of the antiserum to strain S which had been absorbed with elementary bodies of the same strain was lost during the final centrifugation. The part that remained was used for neutralization tests. The results of the examination of these antivaccinial sera are shown in Tables VII, VIII and IX.

It will be seen from the results of the tests with the unabsorbed sera shown in Tables VII and VIII that the antiserum to strain S was slightly stronger than that to strain A, and the elementary body suspension of strain S was more agglutinable than that of strain A, but neither the tests with unabsorbed nor with the absorbed sera indicate qualitative differences in the antigens of the elementary bodies of these two strains.

Comparison of the Cowpox Strains and Vaccinia, Strain S.

The relationship of the two cowpox strains to vaccinia virus strain S have been investigated by the methods of absorption of hyperimmune sera shown

TABLE VII.—*Complement-fixation Tests with the Antisera to Two Vaccinial Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Sera absorbed with E.Bs. of strain—	Antigens. Vaccinia S, Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia A, Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Serum controls. 1/20.	1/40.
Anti-S	Vaccinia A	++++	+	−	−	−	++++	++	±	−	−	+	−
	nil	++++	++++	++++	++	±	++++	++++	++++	+++	±	−	−
Anti-A	Vaccinia S	++	−	−	−	−	+++	+	−	−	−	−	−
	,, A	+++	±	−	−	−	++++	++	−	−	−	−	−
	nil	++++	++++	+++	±	−	++++	++++	++++	++	−	−	−
Normal rabbit	*nil*	−	−	−	..	..	−	−	−	..	..	..	..

Notations as in Table IV.

TABLE VIII.—*Agglutination Tests with the Antisera shown in Table VII.*

Sera.	Sera absorbed with E.Bs. of strain—	E.B. suspensions. Vaccinia S. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia A. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Anti-S	Vaccinia A	±	±	−	−	−	−	−	−	−	−	−	−
	nil	+++	+++	+++	+++	++	+	+++	+++	++	++	+	±
Anti-A	Vaccinia S	−	−	−	−	−	−	±	−	−	−	−	−
	,, A	±	±	−	−	−	−	±	−	−	−	−	−
	nil	+++	+++	+++	++	+	±	+++	++	++	+	±	−
Normal rabbit	*nil*	−	−	−	..	..	..	−	−	−	..	..	..

Notations as in Table V.

TABLE IX.—*Neutralization Tests with Antisera to Two Vaccinia Strains Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Sera.	Sera absorbed with E.Bs. of strain—	Virus in mixtures. Vaccinia S. Dilutions of virus added to 1/5 dilution of sera. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Vaccinia A. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Serum alone.
Anti-S	Vaccinia S	17	10	7	—	—	17	13	9	—	—	—
	,, A	13	9	5	—	—	14	10	—	—	—	—
	nil	4	—	—	—	—	—	—	—	—	—	..
Anti-A	Vaccinia S	14	15	14	7	—	11	8	4	—	—	—
	,, A	15	14	13	6	—	8	7	—	—	—	—
	nil	—	5	—	—	—	—	—	—	—	—	..
Normal rabbit	*nil*	18	19	17	13	4	17	20	18	13	—	..

Notations as in Table VI.

TABLE X.—*Complement-fixation Tests with the Antisera to Cowpox G and Vaccinia S Unabsorbed and After Absorption with the Elementary Bodies of the Two Viruses.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Antigens. Cowpox G. Seitz filtrate. Serum dilutions. 1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia S, Seitz filtrate. 1/20.	1/40.	1/80.	1/160.	1/320.	Serum controls. 1/20.	1/40.
Cowpox G	Cowpox G	±	∓	—	—	—	—	—	—	—	—	—	—
	Vaccinia S	++++	+++	±	—	—	—	—	—	—	—	—	—
	nil	++++	++++	+++	++	∓	+++	+	∓	—	—	—	..
Vaccinia S	Cowpox G	+	∓	—	—	—	++++	+++	++	±	—	—	—
	Vaccinia S	++	±	—	—	—	+	—	—	—	—	—	—
	nil	++++	+++	+	—	—	++++	++++	+++	+	—	—	..
Normal rabbit	*nil*	—	—	..	..	..	—	—	..	..	..	..	..

Notations as in Table IV.

TABLE XI.—*Agglutination Tests with the Sera shown in Table X.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Elementary body suspensions. Cowpox G. Serum dilutions. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Vaccinia S. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.
Cowpox G	Cowpox G	−	−	−	−	−	−	++	+	−	−	−	−
	Vaccinia S	++	++	+	±	−	−	++	++	±	−	−	−
	nil	+++	+++	+++	++	+	±	+++	+++	+++	+++	+	−
Vaccinia S	Cowpox G	−	−	−	−	−	−	+++	+++	+++	+++	+++	+
	Vaccinia S	−	−	−	−	−	−	++	+	−	−	−	−
	nil	+++	++	+	±	−	−	+++	+++	+++	+++	+++	+
Normal rabbit	*nil*	−	−	−	−	..	..	+	±	−	−	..	..

Notations as in Table V.

TABLE XII.—*Neutralization Tests with Sera shown in Table X.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Virus in serum-virus mixtures. Cowpox G. Dilutions of virus added to 1/5 dilution of sera. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Vaccinia S. 10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	Serum alone.
Cowpox G	Cowpox G	27	25	24	12	6	..	24	21	16	15	12	..	−
	Vaccinia S	12	10	4	−	−	..	23	20	12	8	3	..	−
	nil	7	3	−	−	−	..	−	−	−	−	−	..	..
Vaccinia S	Cowpox G	21	27	26	14	4	..	17	−	−	−	−	..	−
	Vaccinia S	22	26	18	11	−	..	18	17	11	8	−	..	−
	nil	7	7	−	−	−	..	−	−	−	−	−	..	..
Normal rabbit	*nil*	31	23	25	14	5	−	21	21	16	15	14	−	..

Notations as in Table VI.

above. The results of the tests with the sera of cowpox strain G and vaccinia strain S are shown in Tables X, XI and XII.

It may be noted that the suspension of elementary bodies of vaccinia used for the agglutination test was slightly serum-sensitive, and the reactions with the lower dilutions of absorbed sera are therefore of doubtful significance. However, it is apparent from the complement-fixation and agglutination reactions with the unabsorbed sera that each serum shows a relatively higher titre with the homologous than with the heterologous antigen. This finding has been constant with different samples of immune sera, and suggests minor antigenic differences between the cowpox and vaccinia S strains. This difference in the unabsorbed sera is not apparent with the neutralization test where the serum concentration is kept constant and is relatively high. The results with the absorbed sera shown in the three tables indicate that the elementary bodies of each strain have absorbed the antibody for both viruses from its homologous serum and its own antibody from the heterologous serum, while only a partial reduction in titre for the other virus has been effected by absorption of the heterologous serum.

Similar results were obtained in corresponding cross-absorption tests with antisera to strains cowpox B and vaccinia S, although in this instance the results were not quite so sharp owing to the difficulty of absorbing the very potent sample of anticowpox B serum used. The results of the neutralization tests with the unabsorbed and absorbed antisera to cowpox B and vaccinia S are shown in Table XIII.

The amount of elementary bodies used to absorb these sera was obviously insufficient for complete absorption, and the suspension of vaccinia virus used for the serum-virus test mixtures had a relatively low titre. Nevertheless the results indicate the same relationship between cowpox strain B and vaccinia strain S as between cowpox strain G and vaccinia S.

DISCUSSION.

The results which have been obtained in cross-immunity experiments on recovered animals with cowpox and vaccinia strains and the results of neutralization tests with unabsorbed sera have confirmed the observations of other workers on the close immunological relationship between the virus of spontaneous cowpox and strains of vaccinia in current use. The closer analysis of the antigens of these viruses by means of absorption of hyperimmune sera with relatively pure suspensions of virus has, however, confirmed the impression gained from agglutination and complement-fixation tests with unabsorbed sera that, although similar, the antigens are not identical.

Serological tests with elementary body suspensions and Seitz filtrates of cowpox virus which had been heated to a temperature of 65° to 70° C. for 1 hour have indicated that this virus, like the vaccinia strains studied by Craigie and Wishart (1934*a* and 1936), and Parker and Rivers (1935), possesses heat-stable and heat-labile components ; but since a detailed examination of these antigens by means of pure " S " and pure " L " antisera has not been made, the difference between the cowpox and vaccinia strains has not been determined in relation to their heat-stable and heat-labile antigenic components.

TABLE XIII.—*Neutralization Tests with Antisera to Strains Cowpox B and Vaccinia S Unabsorbed and After Absorption with Elementary Bodies of the Two Strains.*

Anti-sera.	Sera absorbed with E.Bs. of strain—	Virus in serum-virus mixtures. Cowpox B. Dilutions of virus added to 1/5 dilution of sera. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	Vaccinia S. Dilutions of virus added to 1/5 dilution of sera. 10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.	Serum alone.
Cowpox B	Cowpox B	17	16	19	12	4	..	11	12	5	—	—	..	—
	Vaccinia S	9	—	—	—	—	..	21	14	6	—	—	..	—
	nil	—	—	—	—	—	..	—	—	—	—	—	..	..
Vaccinia S	Cowpox B	24	22	22	17	5	..	—	—	—	—	—	..	—
	Vaccinia S	21	19	18	10	—	..	20	17	10	4	—	..	—
	nil	8	7	—	—	—	..	—	—	—	—	—	..	..
Normal rabbit	*nil*	22	22	21	20	14	9	20	22	20	16	—	—	..

Notations as in Table VI.

Strains of vaccinia virus, such as those in current use, can be produced by adaptation of smallpox virus to the rabbit, the cow or the sheep, but such a virus, as shown by the present work, differs from the virus of spontaneous cowpox. It seems likely that the adaptation of certain pock viruses to the rabbit as achieved by Gins (1919), Van Heelsbergen (1922) and others results in a modification of the antigens of the original strains towards a common structure, but, as Ledingham (1935) points out : " The mere fact that variola cowpox and horsepox can be made to throw off a type of variant possessing apparently similar properties—in fact, a sort of least common denominator which we designate vaccinia—in no way indicates identity of constituent antigen in the serological sense." The two strains of cowpox used in the above experiments have retained their original pathogenicity for the rabbit, and after 14 and 26 passages in this animal species extending over a period of 10 months show antigenic differences from a strain of vaccinia which had been adapted to this animal for a much longer period of time. With more prolonged propagation in the rabbit it is possible that the cowpox strains might become modified, so that the differences between them and the strains of vaccinia might disappear, but so far no evidence of such change has been obtained.

The opinion has been widely held that cowpox is due to infection from a human source, either from cases of variola or vaccinated individuals (see Gins, 1930). The epidemiological evidence, however, shows that, although the spread of infection in milch cows may be effected chiefly by the manipulations of the milkers, " spontaneous " cowpox may occur in cattle without any evidence of contact with variola or vaccinia in man (Frenkel, 1930 ; Blaxall, 1930). In this country cowpox appears to be much more prevalent in certain localities than others in spite of the widespread practice of vaccination of human beings against smallpox. Gins (1938) has recently stated that " für die Existenz einer den Rindern eigentümlichen und selbständigen Pockenkrankheit fehlt auch jetzt noch jeder Beweis ", but the histology of the lesions produced experimentally by strains of cowpox virus as recorded elsewhere (Downie, 1939) and the serological work reported in this paper show that the virus is different from the strains of vaccinia studied, and support the views of Ledingham (1935) and Findlay (1936) that cowpox is to be regarded as a disease *sui generis*.

SUMMARY.

The immunological relationship of two strains of the virus of spontaneous cowpox and two strains of vaccinia virus, one of which was known to have been derived from human variola, have been examined.

Rabbits recovered from infection of either cowpox virus or vaccinia were immune to both, and immune sera prepared against either virus neutralized both.

In agglutination and complement-fixation tests with hyperimmune sera the titres for the homologous virus were usually higher than for the heterologous virus.

By cross-absorption of such sera with elementary body suspensions of the viruses differences in their antigenic composition have been demonstrated.

The results of agglutination, complement-fixation and neutralization tests with the absorbed sera suggest that, although the antigens of cowpox and vaccinia are very much alike, there are qualitative differences in the heat-labile antigens.

These results and the differences in the lesions produced experimentally indicate that the virus of spontaneous cowpox is not the same as the strains of vaccinia virus examined.

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A TYPE OF GROUP A HÆMOLYTIC STREPTOCOCCUS WHICH FAILS TO FORM PEROXIDE.

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THE majority of bacteria form catalase, and hydrogen peroxide cannot therefore be detected in their cultures. The principal exceptions are the anaërobes, the lactic acid bacteria, pneumococci and streptococci. It has been known for some time that hæmolytic streptococci produced peroxide in aërobic culture, but some investigators, *e. g.* McLeod and Gordon (1923), Avery and Morgan (1924), have reported that a few strains were exceptions, forming neither peroxide nor catalase.

No systematic study of the formation of peroxide has appeared since the development of the methods for differentiating the hæmolytic streptococcus into groups and types, and it was not known whether the failure to produce peroxide was the property of a particular group or type.

Two strains of Group A hæmolytic streptococcus, " Richards " and " Robb ", have been much used in the course of recent work on chemotherapy. Both belong to Griffith's Type 3, and when it was found that neither of them produced peroxide, it became of interest to determine whether this was a property of other strains of this type.

TECHNICAL DETAILS.

Methods of Cultivation and of Detection of Peroxide.

(a) *In broth cultures.*

Avery and his associates showed that bacteria form peroxide by the action of a thermolabile enzyme (dehydrogenase), whereby hydrogen is transferred from a hydrogen donator (present in broth) to molecular oxygen. No peroxide is formed in anaërobic culture, and the quantity produced in aërobic culture depends on the oxygen supply, that is, on the surface-volume ratio (Avery and Morgan, 1924). Thus, peroxide production is slow and irregular in deep stationary cultures, but is increased in shallow layer cultures which expose a large surface to the air. We have found the greatest and most consistent formation of peroxide in cultures in trypsinized heart-broth containing 20

per cent. horse-serum, shaken during incubation in a suitable agitator (Willcocks, 1938). In a shaken culture the effective surface-volume ratio is very large.

Peroxide was determined in these cultures by the following method, which was worked out by Mr. K. Borthwick Taylor at these laboratories :

One c.c. of culture is mixed with an equal volume of buffer (pH 4·0) ; 5 drops of 1 per cent. tolidine (B.D.H.) in 50 per cent. alcohol and 1 drop of peroxidase extract* are added. A blue colour develops in the presence of peroxide. It is advisable to dilute the test liquid until it contains between 1 in 10,000 and 1 in 100,000 of 20 vol. peroxide, for if the peroxide is stronger than this the colour is transitory.

This method will detect peroxide down to a dilution of 1 in 500,000 (0·0002 per cent.) of 20-vol. peroxide, or 1 in 3 million of absolute peroxide. For approximate quantitative work, the test sample may be compared with standards prepared from dilutions of hydrogen peroxide, or permanent standards may be made from methylene-blue. An aqueous solution of 2·5 mg. per c.c. gives a colour equivalent to 1 in 10,000 of 20-vol. peroxide.

With this method Mr. Taylor has detected peroxide in sterile infusion and digest broth. The source of the peroxide is the meat, not the added peptone. It is destroyed by boiling, but reappears in 12 hours.

(b) *On blood-agar plates.*

Plates of ordinary nutrient blood agar are poured, and before use a small quantity of an alcoholic solution of benzidine is poured over the surface and allowed to dry. On this medium colonies which have produced peroxide are stained blue, black or brown (Penfold, 1922). The colour appears first at the apex of the colony, that is, farthest away from the blood catalase.

To avoid the possibility that the catalase in the blood plate was preventing the detection of small amounts of peroxide, nutrient agar plates with the addition of horse-radish peroxidase and benzidine were used, but they seemed to have no advantage over the benzidine blood plates. Tolidine blood-plates were not so satisfactory as the benzidine plates.

(c) *On heated blood-agar plates.*

Blood-agar which has been heated to 80° C. to destroy the catalase is bleached by peroxide. Colonies not forming peroxide do not affect the medium in any way (McLeod, Gordon and Pyrah, 1923).

Preparation of Catalase.

1. Dry catalase was prepared by adding an equal volume of alcohol to horse-blood, filtering off the precipitated proteins, drying them *in vacuo*, extracting the catalase from them with water at 0° C., and evaporating the extract to dryness *in vacuo*.

2. Blood-cells were treated with chloroform and alcohol, the precipitated

* The peroxidase extract is an aqueous extract of horse-radish, which has previously been extracted with alcohol to remove oxidases.

proteins spun down, and the organic solvents removed from the clear colourless extract, *in vacuo*. This extract contained approximately the same concentration of catalase as the original blood.

3. Pig liver was minced, mixed with an equal volume of dioxane, filtered, and the catalase precipitated with an equal volume of alcohol. The precipitate was dried *in vacuo*, and the catalase extracted with water. The last two preparations were filtered through a Seitz before use.

EXPERIMENTAL.

Results of the Peroxide Tests.

Seventy-five strains from all Griffith's Types 1–29 were tested, with the exception of Types 7, 16, 20 and 21, which do not belong to Group A. All were examined after 24 hours in shaken serum broth culture and on benzidine blood-plates. Some were also tested by growing for 24 hours on heated blood-agar.

Type 3 strains.

Ten Type 3 strains—" Richards ", " Robb ", " Teverson ", " Vennell ", " Leckie ", " Chattington ", " Lewis ", " Ridley ", " Pidgeon " and P. 279—were used. All failed to show peroxide in shaken serum broth culture, on benzidine blood-agar or on heated blood-agar. Some of these strains have been tested repeatedly during the past two years, always with negative results.

Todd has shown (1930) that batches of broth vary greatly in their power to destroy peroxide. Cultures of Type 3 in broth which had been chosen for absence of peroxide-destroying power have not produced detectable amounts of peroxide.

No catalase has been demonstrated in the cultures, for bubbles of oxygen could not be detected when broth cultures or colonies on agar were treated with hydrogen peroxide. In mixed cultures of Type 3 and peroxide-positive types, the presence of the Type 3 organisms did not prevent the formation of peroxide.

Strains of other types.

Of the other 65 strains only 2 (one Type 1 and one Type 5) failed to form peroxide. Since 14 other Type 1 and 2 other Type 5 strains produced peroxide, and the 2 negative strains (SF 130/2 and " Franklin ") were not exhaustively tested, *e. g.* in specially selected broth, it is possible that under different conditions they might have proved peroxide-positive.

By their failure to form peroxide, Type 3 strains are thus sharply differentiated from other Group A strains.

Other Distinguishing Characters of Type 3 Strains.

(a) *Digestion of human fibrin.*

Type 3 strains differ from other types in their fibrinolytic power. Most Group A streptococci digest susceptible human fibrin in 15–30 minutes in the

Tillett-Garner test (Tillett and Garner, 1933). All Type 3 strains, on the other hand, dissolve human fibrin slowly. The quickest reaction we have obtained was in 90 minutes, while the majority of strains took from 2½ to 12 hours. Occasionally a strain from the other types has a weak fibrinolytic power, but only in Type 3 is the fibrinolysin so consistently weak.

(b) *Action of catalase.*

Many hæmolytic streptococci produce peroxide in shaken cultures to such an extent that the culture sterilizes itself in 12–24 hours. If catalase is added to the culture-medium, these strains survive for 48 hours or more. We were surprised to find, on the other hand, that Type 3 strains were inhibited from growing by catalase, strain " Richards " being killed by as little as 0·1 per cent. of the dry catalase. Table I illustrates the different effects of catalase in broth culture on " Turner " (a vigorous peroxide producer), whose growth it prolongs, and on " Richards " (Type 3), which is killed by the higher concentration of catalase.

TABLE I.—*Showing the Action of Catalase in Prolonging the Life of Peroxide-forming Cultures and Inhibiting the Growth of Type* 3.

Strain.	Implant.	Population at 18 hours in serum broth containing catalase as under (per cent. dry weight).			
		0.	0·001.	0·01.	0·1.
" Turner "	8,800	0	4500	2×10^6	..
,,	11,400	4000	..	5×10^6	$3{\cdot}5 \times 10^6$
" Richards "	11,400	$10{\cdot}6 \times 10^6$	..	33,000	0

On nutrient agar plates containing in addition 2 per cent. of liver catalase extract, peroxide-producers grow luxuriantly both aërobically and anaërobically. Type 3 strains were unable to grow aërobically on these catalase agar plates. With weaker catalase extracts growth was delayed, and small colonies appeared after 48 hours' incubation. When the plates were incubated anaërobically, the catalase was without effect on the growth of Type 3 strains—they grew equally well in presence or absence of catalase.

We are unable to explain this difference in the effect of catalase under aërobic and anaërobic conditions. Catalase is present in most species of bacteria, although absent from streptococci, and no other function for it has been suggested apart from preventing the accumulation of bacterial peroxide. The catalase used was not pure, but contained other enzymes. The inference that the action was due to the catalase may be made from the fact that the stronger the catalase activity of the preparations, the more marked was their effect.

DISCUSSION.

The difference between Type 3 and the other types of Group A hæmolytic streptococci is so striking that it is possible that a profound difference in metabolism exists. Besides their non-production of peroxide and sensitivity

to catalase, they also digest human fibrin more slowly than the other types. No attempt has been made to examine their fermentation reactions, etc., quantitatively, or to find whether they form a sub-group antigenically. Type 3 strains grow as vigorously as the other types in the ordinary laboratory media, so the non-production of peroxide and slow fibrin digestion cannot be due to a less robust growth.

The inhibition of growth on catalase-agar plates is interesting, for no inhibition occurs on blood-agar plates, which contain large amounts of catalase. Some constituent of the blood may counteract the effect of the catalase.

It has been suggested by Shinn, Main and Mellon (1938) that the chemotherapeutic action of sulphanilamide is due to the anti-catalase action of this substance and its oxidation products. Their view is that in presence of sulphanilamide the bacterial and host-tissue catalase is inhibited, thus allowing toxic quantities of peroxide to accumulate.

An apparently insuperable objection to this theory is that the strains " Richards " and " Robb ", which are universally recognized as being susceptible to sulphanilamide, do not produce peroxide.

SUMMARY.

Type 3, Group A, streptococci are distinct from all other types tested in not producing peroxide. Their growth in aërobic but not anaërobic culture is inhibited by catalase. They digest human fibrin more slowly than do the other types of Group A.

We wish to thank Dr. L. Colebrook for his help during this work.

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THE SIZE OF THE VIRUS OF LYMPHOCYTIC CHORIOMENINGITIS AS DETERMINED BY ULTRAFILTRATION AND ULTRACENTRIFUGATION.

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SCOTT and Rivers (1936) reported the isolation of the virus of lymphocytic choriomeningitis from the spinal fluids of two patients suffering from acute lymphocytic meningitis. They showed that the virus isolated from one case was identical in every respect with that isolated from the other. Preliminary work done at that time suggested that the size of the virus particles was between 100–150 mμ. in diameter. Since then ultrafiltration and ultracentrifugation studies have been directed towards obtaining a more accurate estimate of the virus particle size and the results are reported in this paper.

The prosecution of these studies has involved surmounting certain inherent difficulties connected with the manipulation of this virus, which must be herewith described before the experimental results themselves can be clearly presented. It was pointed out in the earlier paper that this virus may not kill all of the infected mice, even when an undiluted 10 per cent. brain emulsion is given intracerebrally.

The few mice that recover from an inoculation of this strength usually show obvious signs of illness, such as sitting hunched up and motionless with roughened fur. An occasional mouse, however, may become infected and recover without ever showing clinical evidence of disease. With higher dilutions of the virus such subclinical infections become more frequent, so that it is impossible by mere observation of a group of inoculated mice to distinguish those subclinically infected from those escaping infection altogether. The only way to make this distinction seemed to be the demonstration of immunity in the mice that had been subclinically infected, since it was known that even subclinical infections in this host produced a complete resistance to reinoculation with potent virus material. The routine technique used in the present experiments therefore has been as follows :

* Personal grant from Medical Research Council aided part of this work, which includes part of a thesis submitted for M.D. degree at Cambridge.

METHODS.

The mice were given an original inoculation of the material to be tested and watched for three weeks. The number dying within a period of 5–21 days after this inoculation were noted as being due to specific infection, in view of the experience of Wooley, Armstrong and Onstott (1937), who found that animals dead within that period after inoculation practically always died of the infection, and not accidentally. On the 17th–18th day, after the primary inoculation, the surviving mice were re-inoculated intracerebrally with 0·025–0·03 c.c. of 10 per cent. emulsion of virulent mouse brain. Mice dying on the day of or the day after the second inoculation, were discarded as dying from trauma, and not from infection. Animals dying between 1 and 4 days after second inoculation were considered as dying from the previous inoculation. (Justification for this reasoning may be found in the work of Traub (1936), who had noted that when mice infected spontaneously by the natural disease were inoculated intracerebrally with a known emulsion of virus, some became typically ill and died within 2–5 days, while the control mice succumbed after the usual incubation period of 6–8 days.) The animals dying after the 5th day were considered as being previously uninfected, while the immunity shown by those that remained perfectly well for 14 days after the second inoculation was considered as evidence of previous infection by the primary inoculation.

TABLE I.—*Experiment Illustrating the Difference in Titre Obtained in Mice Receiving an Original Intracerebral Inoculation only and an Original Inoculation by Both Intracerebral and Intraperitoneal Routes.*

Intracerebral inoculation only.

Dilution.	Number dying after original inoculation.	Number surviving re-inoculation.	Total number infected.
10^{-1}	5/6	1/1	6/6
10^{-2}	0/6	0/6	0/6
10^{-3}	0/6	0/6	0/6
10^{-4}	1/6	0/4 (1 accidental death)	1/6
10^{-5}	0/6	0/6	0/6

50 per cent. end-point between 10^{-1} and 10^{-2}. Titre = 1 : 38.

Intracerebral and intraperitoneal inoculation.

Dilution.	Number dying after original inoculation.	Number surviving re-inoculation.	Total number infected.
10^{-1}	5/6	1/1	6/6
10^{-2}	0/6	5/6	5/6
10^{-3}	0/6	2/6	2/6
10^{-4}	0/6	0/6	0/6
10^{-5}	0/6	0/6	0/6

50 per cent. end-point between 10^{-2} and 10^{-3}. Titre = 1 : 430.

Note.—Fractions written with number mice dying and surviving respectively as numerator and the total number of mice inoculated as denominator.

Comment.—The titre by intracerebral method of inoculation is 1/10 that of combined method.

The number of animals, therefore, considered as being infected by the material under test was represented by the sum of the animals dying from the primary and surviving the secondary inoculation.

The concentration of virus was measured by titrations in mice, the titre being taken to be that dilution of virus at which 50 per cent. of the animals were infected and 50 per cent. uninfected. This method has been shown by Reed and Muench (1938) to yield a most satisfactory accuracy in measuring titration end-points.

In all filtration experiments, the titre of the stock solution to be filtered was estimated. The virus contained in the membrane filtrate was titrated in a few instances, but generally its presence or absence only was recorded. In the centrifugation experiments the supernatants and unspun controls were titrated.

The titres of the various samples of virus used varied considerably and were, on the whole, low. Experience showed that higher titres resulted when mice were inoculated both intracerebrally 0·03 c.c and intraperitoneally 0·5 c.c. This dual intraperitoneal-intracerebral inoculation raised the titre as much as 10-fold on occasions as compared with intracerebral inoculation only (see Table I).

The combined intracerebral-intraperitoneal inoculation technique was employed for the last five filtration experiments and in all the centrifugation studies.

THE SIZE OF THE VIRUS FROM ULTRAFILTRATION.

It has already been recorded (Scott and Rivers, 1936) that the virus passed readily through V., N., W., Berkefeld candles and with difficulty through a Seitz asbestos pad. The filtrations here described were made through graded collodion membranes of known pore size (Elford, 1931).

Material and Methods.

The brains of mice dying spontaneously, or killed at the height of illness following intracerebral inoculation of virus, were removed aseptically. A 2–3 per cent. emulsion of bacteriologically sterile brains was prepared by grinding a weighed quantity of the tissue in the chosen medium, which for the first eight experiments was: Nutrient meat infusion broth, 50 per cent.; human ascitic fluid, 25 per cent.; distilled water, 25 per cent. This medium had a pH of 7·34 by glass electrode. Full nutrient meat infusion broth was used in the last four experiments. The emulsion was centrifuged at 2000 revs./min. on the International centrifuge for 15–20 minutes, and the supernatant decanted off. In the first eight experiments this supernatant was filtered through a "V" Berkefeld candle—through which 50 c.c. broth had previously been passed—and then through the test membranes. In the last four experiments the supernatant was cleared by passage through an asbestos pulp filter, which had first been saturated with nutrient broth. The clarified filtrate was then passed through a membrane 750–790 mμ. A.P.D., the resulting filtrate serving as the stock for subsequent analyses with finer porosities.

Experimental Results.

The results of twelve experiments in which the stock filtrates (from Berkefeld "V" candle and from 750 mμ. [*ca.*] membranes) were passed under 15 lb./sq. in. pressure through membranes of progressively smaller porosities are summarized in Table II.

TABLE II.—*Membrane Filtrations.*

Titre of Stock filtrate.	Average pore size of membranes in millimicra. 195 and above.	170.	160.	150.	140.	130.	120.	110.	100.	60.
(*a*) 1 : 3,300	+	+	−	+	−	−	−	−	−	−
(*b*) 1 : 3,300	−	−	−	+	O	O	O	O	O	−
(*c*) 1 : 2,300	−	−	−	+	+	+	O	O	O	−
(*d*) 1 : 1,750	−	−	−	O*	+	O	O	O	O	−
(*e*) 1 : 3,300	−	−	−	O	O	O	O	−	−	−
(*f*) 1 : 1,750	−	−	−	O	O	O	O	−	−	−
(*g*) 1 : 5,800	−	+	−	+	O	+	O	−	−	−
(*h*) 1 : 33,000	+	+	−	+	+	+	+	−	−	−
(*i*) 1 : 3,000	+	−	−	−	±	−	−	−	O	O
(*j*) 1 : 430	+	−	+	−	±	−	−	O	−	−
(*k*) 1 : 2,775	+	−	+ (1 : 25)†	−	±	−	−	O	−	−
(*l*) 1 : 3,150	+	−	+ (1 : 32)	−	±	−	−	O	−	−

+ = Virus demonstrated. ± = Doubtful demonstration of virus. O = No virus demonstrated. − = Membrane not used. * = Probably two membranes used in error. † = Figures in brackets indicate the titre of membrane filtrate.

The table clearly shows two things: (1) the marked variability of the titre of the stock filtrate already mentioned; and (2) that the limiting membrane porosity for retention of the virus under the given filtration conditions depends on the inital titre of the stock filtrate. This phenomenon has been pointed out previously by Elford and Andrewes (1932), who stressed the necessity of using the highest concentration of virus attainable in order to get a reliable filtration end-point. As can be seen from the table, only on one occasion was a really high titre obtained. Nevertheless, the data provided by these experiments are sufficient to enable an estimate of the approximate particle size of the virus to be made.

The evidence can be presented as follows: With stock titres up to 1 : 3000 virus was demonstrated in the filtrates through membranes of A.P.D. 140 mμ. in 6 out of 9 attempts. Of the 6 positive filtrates there was no doubt about the presence of virus in 2, but in the 4 marked as doubtful the mice showed infection in an irregular fashion; for instance, mice would be infected by a dilution of 10^{-1}, but not by the undiluted filtrate. Occasionally mice would be infected in 10^{-2} and 10^{-3} dilutions where the lower dilutions failed to cause infection. The explanation of this phenomenon awaits further investigation, but it is interesting to note that Bengston (1936), while cultivating Armstrong's strain of virus on the chorio-allantoic membrane of hen's eggs, noted a similar phenomenon when the virus was growing poorly. It suggests the possible

presence of an antiviral agent, demonstrable only when the virus is very weak, and which may soon be diluted out beyond its effective concentration, or which may be dissociated from combination with virus by dilution.

With a titre of almost 1 : 6000, virus was demonstrated in the filtrate of membrane 130 mμ. A.P.D. in the one attempt made, while, when the titre was 1 : 33,000, virus was demonstrated in filtrates of membranes down to and including 120 mμ. Virus has never been demonstrated in the filtrate through membranes of A.P.Ds. smaller than 120 mμ., although it must be pointed out that in none of six stock filtrates used in such experiments was the virus titre higher than 1 : 3300.

The evidence furnished by these studies points to the true filtration end-point being close to 100 mμ. This is interpreted as indicating a probable particle size for the virus 40–60 mμ. (see Elford, 1933, 1937).

THE SIZE OF THE VIRUS BY CENTRIFUGATION.

Two techniques of centrifugation analysis were applied, both of which have been previously described in detail (Elford, 1936), so they will only be very briefly outlined here.

(1) The method due to Bechhold and Schlesinger (1931), in which the material under investigation is centrifuged at high speed in a flat-bottomed cylindrical glass tube, 5 cm. in length by 1 cm. in width, at the bottom of which is placed a disc of thick filter-paper serving to retain particles which sediment, and thus minimizing any tendency to redispersion. Schlesinger (1934) found that the conditions prevailing during spinning in such wide tubes justified the assumption of complete mixing in the supernatant fluid. Accordingly by considering the rate at which particles are removed from the supernatant into the filter-paper, he deduced the following relationship permitting the particle diameter, d, of a suspension to be calculated from the determined ratio of the initial concentration, C_o, to the average concentration, C_t, in the supernatant after spinning for time, t. Thus—

$$d\ (\text{m}\mu.) = 6{\cdot}15 \times 10^8 \sqrt{\frac{\eta\ \text{h.}\ C_o/C_t}{(\sigma_p - \sigma_m).\text{R.T.N}^2}} \quad . \quad . \quad . \quad (1),$$

where σ_m and η are respectively the density and viscosity of the medium.

σ_p = density of the suspended particles.
h = height of the liquid column above the filter-paper disc.
R = distance of the filter-paper disc from the axis of rotation.
T = time of spinning in secs.
N = speed of centrifuge in revs./min.

(2) The inverted capillary-tube technique devised by Elford (1936) enables the sedimentation to be measured under conditions for which Stoke's law may be directly applied. The rate at which the particles leave an inverted capillary tube, 1 cm. long by 1 to 2 mm. in diameter, is determined, the initial concentration, C_o, of the suspension and the average concentration, C_t, within the capillary after centrifuging for the time, t, being measured. The relationship enabling the particle diameter, d, to be calculated is—

$$d\ (\mathrm{m}\mu.) = 7{\cdot}94 \times 10^{7} \sqrt{\frac{\eta \log. \dfrac{x+l}{x+l\ C_l/C_o}}{(\sigma_p - \sigma_m)\ N^2\ .\ t}} \quad . \quad . \quad (2),$$

where t is the time of spinning expressed in minutes.

l = length of the capillary in cm.

x = distance in cm. of the upper closed end of the capillary from the axis of rotation.

Other symbols have the meaning as in equation (1).

Experimental Results.

Our experiments have been made with stock membrane filtrates of the virus from infected mouse brain tissue contained in a broth medium of known density and viscosity. The various systems before and after spinning were titrated according to the technique already described in the filtration section. The centrifuge employed was the " Ecco Blitz " model of Collatz, and capable of 15,000 revs./min., providing a maximum centrifugal force 20,000 g.

The experimental data are best summarized in tabular form and are contained in Tables III and IV.

TABLE III.—*Centrifugation Data for the Virus of Chorio-meningitis by Method of Bechhold and Schlesinger.*

Expt.	Medium.	σ_m.	η.	Speed (r./m.).	Time (mins.).	h (cms.).	R (cms.).	C_o/C_t.	Calculated particle diameter, d, in mμ.
I	Lemco broth	1·009	0·009	10,000	60	1·3	8	2·13	52
II	Ditto	1·009	0·009	9,000	60	1·4	8	1·33	37·5

TABLE IV.—*Centrifugation Data for the Virus of Chorio-meningitis by the Inverted Capillary-tube Method.*

Expt.	Medium.	σ_m.	η.	Speed (r./m.).	Time (mins.).	x.	C_t/C_o.	Calculated particle diameter, d, in mμ.
I	Ox-heart broth	1·013	0·0103	8600	60	1·0	7·25	0·42	47
II	Ditto	1·013	0·0103	9500	60	1·0	7·25	0·055	55

N.B.—Density of virus particle σ_p assumed to be 1·20 in all calculations of d.

The interpretation of centrifugation data requires that the density of the particles as well as that of the medium in which they are suspended be known, *cf.* equations 1 and 2. The value 1·20 has been assumed for the density of chorio-meningitis virus in calculation d in Tables III and IV. The evidence at present available as to the densities of viruses would indicate 1·20 to be a fair average value. Should the true density of this virus differ from 1·20 by $\pm$ 0·05, then a correction amounting to — 10 or + 15 per cent. respectively would need to be applied to the calculated particle diameter.

It is further implicity assumed in the above calculations that the virus

particles are spherical and homodisperse. These factors merit extended investigation when more potent material is available.

CONCLUSION.

The evidence provided by the centrifugation studies indicates the particle size of the infective principle of chorio-meningitis to lie between 37 and 55 mμ., while the method of ultrafiltration analysis has given the probable size as 40–60 mμ. These findings by two independent methods, considered in the light of the experimental difficulties attending work with the particular virus concerned, must be held to show very satisfactory agreement.

We wish to acknowledge our gratitude to Dr. J. H. Bauer of the Rockefeller Foundation for his help in the earlier filtration experiments, which were performed in the laboratories of the Rockefeller Institute, New York.

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INTER-RELATIONSHIPS BETWEEN AMINO-ACIDS IN THE NUTRITION OF *B. ANTHRACIS*.

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In the course of an investigation into the amino-acid growth requirements of *B. anthracis*, an inter-relationship between groups of amino-acids was discovered which has not so far been described in metabolic studies. This work forms the subject of this paper.

TECHNICAL DETAILS.

All the precautions required in bacterial metabolic studies described in the earlier papers of Fildes and his colleagues were observed.

The Medium.

The medium was a modification of that used by Fildes, Richardson, Knight and Gladstone (1936). It contained in its complete form the following amino-acids: †s-aspartic acid 0·2 g., s-valine and s-leucine 0·15 g., s-alanine, s-glutamic acid‡, s-*iso*leucine, s-phenylalanine and s-lysine HCl 0·1 g., s-glycine, *l*(−)-proline, *l*(−)-oxyproline, *l*(−)-tyrosine§, *l*(+)-arginine HCl and *l*(−)-histidine HCl 0·05 g. These quantities were dissolved in 600 ml. of *M*/18 phosphate buffer (4·5 g. KH_2PO_4 + 26 ml. *N*/1 NaOH), adjusted to pH 7·4, tubed in 6 ml. and autoclaved. To each tube was added: *l*(−)-cystine *M*/200 in *N*/10 HCl (Seitz-filtered) 0·2 ml., NaOH *N*/5 (autoclaved) 0·1 ml., s-methionine *M*/100 (Seitz-filtered) 0·2 ml., *l*(−)-tryptophan *M*/200 (autoclaved) 0·1 ml., glucose *M*/2 (Seitz-filtered) 0·25 ml., $MgSO_4 7H_2O$ *M*/60 (autoclaved) 0·1 ml., ferrous ammonium sulphate *M*/500 in *N*/50 HCl (Seitz-filtered) 0·25 ml. and water to 10 ml. When it was desired to vary the concentration of any one or more amino-acids in the medium, such amino-acids were excluded from the basal medium and sterilized separately in a concentration × 100 of that in the complete medium; the addition of 0·1 ml., therefore, gave the original concentration. It will be noticed that the concentrations of most of the amino-acids have no molecular basis. Aliquot quantities, as near as possible to the molecular concentrations of Fildes' medium, were used in order to

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† The prefix "s" denotes an amino-acid of synthetic origin.

‡ Synthesized by the method of McIlwain and Richardson (1939).

§ In later experiments s-tyrosine was used. In order to keep the molar concentration of one only of the optical isomers of the racemic mixture constant, twice the concentration (0·1 g.) was used.

facilitate rapidity in weighing. Where an amino-acid was tested for its effect on growth, its concentration was always expressed as a molarity. It was found necessary to modify the concentration of certain amino-acids in later experiments; such modification is stated later in the paper.

Where indicated, in addition to the amino-acids listed above, solutions of the following were used in the concentrations stated later: s-threonine (α-amino-β-hydroxybutyric acid) (autoclaved), s-serine (Seitz-filtered), s-*nor*-leucine (autoclaved), s-α-amino-butyric acid (Seitz-filtered), s-β-hydroxy-butyric acid (Seitz-filtered) and s-β-hydroxyglutamic acid (Seitz-filtered). In experiments in which natural preparations of leucine, *iso*leucine and valine were used, these amino-acids were as follows: *l*(+)-leucine (British Drug Houses) recrystallized three times from 60 per cent. alcohol, *l*(+)-*iso*leucine (Hoffmann-La Roche) not recrystallized, and *l*(+)-valine (Hoffmann-La Roche) recrystallized three times from 60 per cent. alcohol, treated with HCl to remove all insoluble hydrochlorides, treated with pyridine and recrystallized from water. Glutamine, a necessary factor for the growth of *Streptococcus pyogenes* (McIlwain, Fildes, Gladstone and Knight, 1939), was found to increase the rate of growth of *B. anthracis* to a small extent. In later experiments it was therefore included; 0·25 ml. of *M*/50 *l*(+)-glutamine (Seitz-filtered) was added per tube.

It may be emphasized that the medium contained no substance whose chemical composition was not known.

The Strains of B. anthracis *used.*

The strains of *B. anthracis* were all from the National Collection of Type Cultures. They were as follows:

1. "Armitage"	N.C.T.C. No.	1607
2. From Hankow hide	,,	2620
3. From Chinese toothbrush	,,	945
4. "Vaccin Charbonneux No. 2"	,,	1712
5. "Exeter"	,,	110
6. "James" (avirulent)	,,	631
7. "A2723"	,,	4991
8. "Vaccin Charbonneux No. 1"	,,	1711

Inoculum.

Except in "training" experiments, spore suspensions were used throughout. Their preparation was similar to that used by Knight and Fildes (1933) for *Cl. sporogenes*. One drop of a just visible suspension was inoculated per tube.

Incubation.

All cultures were grown at 37° C. in jars containing 5 per cent. CO_2 in air.

EXPERIMENTAL.

All strains of *B. anthracis* except strains 2 and 4 grew well on the complete medium. Strain 6 grew in two days; the others grew overnight (18 hours), and

gave a maximum heavy growth in 24 hours. With the exception of strain 8, which produced a pellicle, all strains gave an initial diffuse growth, which later became granular.

Using the technique adopted in the study of the amino-acid growth requirements of *Staphylococcus aureus* (Gladstone, 1937) of leaving out single amino-acids from the complete mixture, it was found that growth was considerably affected by the removal of one of the following: leucine, valine, *iso*leucine, glycine or cystine. The removal of leucine completely prevented the growth of strains 5, 6 and 7 (9 days), whilst that of strains 1 and 3 was delayed to the second day. The growth of strain 8 was only slightly affected. The effect of removing valine was even more marked; no growth occurred with strains 3, 5 and 7, whilst strains 1 and 6 only grew after 4 days and strain 8 only after 2 days. With all strains the removal of *iso*leucine, cystine or glycine did not abolish growth but delayed it, and it remained less than that produced in the complete medium (Table I). The removal of other amino-acids singly had no appreciable effect on growth, except for a slight decrease in the rate of growth of strains 1 and 6, when tryptophan was removed.

TABLE I.—*The Effect on the Growth of* B. anthracis (*Strain* 5) *of Omitting Single Amino-acids from the Complete Medium.*

Medium.	Growth.					
	Hours.		Days.			
	16.	40.	3.	4.	6.	9.
Complete medium .	+	+++	+++	+++	++	++
No leucine . . .	0	0	0	0	0	0
No valine . . .	0	0	0	0	0	0
No *iso*leucine . .	0	++	++	++	++	+
No glycine . . .	0	tr.	+	++	++	+
No cystine . . .	0	0	++	++	++	+

Note.—0 = no growth, tr. = trace; *plus* signs are proportional to the amount of growth as judged by the degree of opacity.

From the results recorded in Table I, it was at first assumed that valine, leucine, *iso*leucine, glycine and cystine were all required for growth, but that the last three could be synthesized to some extent by the organism allowing late growth in their absence. Further tests showed that this statement could only be applied directly to glycine and cystine, the removal of which under all conditions of test adversely affected growth. Glycine appeared to be required in a somewhat larger quantity than that present in the medium of Fildes *et al.* (1936). In subsequent tests it was added in a concentration of $M/300$.

Valine, Leucine and Isoleucine.

Inhibitory effect of each singly on growth.

In a test designed to confirm the necessity for these amino-acids, a control culture was included in which all three were absent. Since no growth occurred in the absence of valine or leucine and only delayed growth in the absence of *iso*leucine, it was naturally assumed that growth would be entirely absent

when all three were excluded. It was, therefore, surprising to find that growth started after a delay of 2 days, and reached a maximum rather less than that in the complete mixture. Further tests showed that the addition of any one of these three amino-acids to a mixture in which they were all absent completely abolished this growth. Table II shows the smallest quantity of each amino-acid which effects this inhibition.

TABLE II.—*The Effect on Growth (Strain 5) of Adding Different Concentrations of Valine, Leucine or* Iso*leucine singly to a Medium lacking all Three.*

Medium.		Growth.				
		Hours.			Days.	
		13.	22.	38.	3.	4.
Amino-acid medium without valine, leucine or *iso*leucine		0	0	+	++	++
Ditto, with valine	*M*/1500*	0	0	0	0	0
,, ,,	*M*/7500	0	0	0	0	0
,, ,,	*M*/37,500	0	0	0	0	0
,, ,,	*M*/187,500	0	0	0	0	0
,, ,,	*M*/937,500	0	0	0	++	++
,, ,,	*M*/4,687,500	0	0	0	++	++
,, ,,	*M*/23,437,500	0	0	tr.	++	++
,, with leucine	*M*/1700	0	0	0	0	0
,, ,,	*M*/8500	0	0	0	0	+
,, ,,	*M*/42,500	0	0	0	0	+
,, ,,	*M*/212,500	0	0	0	+	++
,, ,,	*M*/1,062,500	0	0	0	++	++
,, ,,	*M*/5,312,500	0	0	+	++	++
,, with *iso*leucine	*M*/2500	0	0	0	0	0
,, ,,	*M*/12,500	0	0	0	0	0
,, ,,	*M*/62,500	0	0	0	0	0
,, ,,	*M*/312,500	0	0	0	0	0
,, ,,	*M*/1,562,500	0	0	0	+	++
,, ,,	*M*/7,812,500	0	0	+	++	++
Complete amino-acid medium with valine *M*/1500, leucine *M*/1700 and *iso*leucine *M*/2500		tr.	+++	+++	+++	+++

* Since all three amino-acids are of synthetic origin, the molarities were calculated in terms of one only of the optical isomers of the racemic mixtures.

*Iso*leucine was the most toxic for growth (down to a concentration of *M*/312,500), then valine (*M*/187,500), and finally leucine (*M*/42,500). In all cases, especially with leucine, toxicity became less marked as time of incubation was prolonged. The organism appeared to be able to overcome to some extent the inhibitory effect provided sufficient time was allowed. This was further established when the titrations (Table II) were repeated, using as inocula cultures growing late in the presence of an initially toxic concentration of one of the three amino-acids. Growth occurred in still higher concentrations of

this amino-acid. On repeating the process, the organism was able to grow with little delay in a mixture containing five times the highest concentration of the amino-acid shown in Table II. Cultures " trained " in this way to overcome the inhibitory effect of valine grew also in media containing inhibitory concentrations of leucine or *iso*leucine, but the effect was less pronounced. Cultures trained to grow with toxic concentrations of *iso*leucine, however, failed to grow in the presence of valine alone, but were able to grow in the presence of an initially toxic concentration of leucine alone. The training process was therefore not strictly specific.

Mutually antagonistic effect on toxicity and growth-stimulating effect.

From Table II it can be seen that, when all three amino-acids, leucine, valine and *iso*leucine were present, inhibition was not only abolished, but growth was better and quicker than in their absence. They must, therefore, be capable of not only mutually antagonizing each other's toxic effects, but, having done so, of actually stimulating growth. It remained to discover whether all were equally concerned in this process.

In Table I, if the results obtained when valine, leucine or *iso*leucine was omitted, are regarded not from the point of view of the omission of one amino-acid, but of the addition of the other two, the absence of growth when leucine and *iso*leucine and when valine and *iso*leucine were present can be explained as the failure of each of these amino-acids to " neutralize " the toxicity of the other in the pair. In the presence of valine and leucine together, however, growth was obtained. Although it is inferior to that of the complete mixture, it is more rapid than when all three amino-acids are excluded. The toxic effect of valine and leucine is, therefore, counteracted by each other, but that of *iso*leucine is only neutralized when both valine and leucine are present together. This is summarized in Table III.

TABLE III.—*Summary of the Effect of Valine, Leucine and* Iso*leucine Singly and in Combination on the Growth of* B. anthracis (*Strain* 5). *Basal Medium Lacking Valine, Leucine and* Iso*leucine.*

				Growth.				
				Hours.			Days.	
Valine.	Leucine.	*Iso*leucine.	16.	22.	40.	3.	4.
Basal medium +	0*	0	0	0†	0	+	++	++
Ditto	+	0	0	0	0	0	0	0
,,	0	+	0	0	0	0	0	0
,,	0	0	+	0	0	0	0	0
,,	0	+	+	0	0	0	0	0
,,	+	0	+	0	0	0	0	0
,,	+	+	0	0	+	++	++	++
,,	+	+	+	+	+++	+++	+++	+++

* 0 denotes absence, + presence, of the amino-acid heading each column; quantities as in the complete amino-acid medium.

† Here, as in other tables, 0 = no growth, and *plus* signs are proportional to the amount of growth.

Quantitative relations.

It is not practicable to investigate with any degree of accuracy the quantitative relations between three variables together. The method adopted, therefore, was to investigate first the relations between leucine and valine in the absence of *iso*leucine. The smallest amount of valine which would "neutralize" the toxicity of $M/1700$ leucine was $M/37{,}500$, and the smallest quantity of leucine "neutralizing" the toxicity of $M/1500$ valine was $M/42{,}500$. Growth at these limiting figures was poor and delayed. For optimum growth it was necessary that the molar ratio between the concentration of valine and leucine should be approximately 1 : 4·5, and that the concentrations of each should not be less than $M/7500$ valine and $M/1700$ leucine. With these concentrations of valine and leucine, the optimum concentration of *iso*leucine was found to be approximately $M/2500$, *i. e.* about three times the molar concentration of valine. With valine, leucine and *iso*leucine combined in these proportions, it was found that the smallest quantity producing growth in 17 hours was $M/468{,}750$ valine, $M/106{,}250$ leucine, and $M/156{,}250$ *iso*leucine, but even when the concentrations were reduced to about $M/1 \times 10^{-9}$, slight acceleration in growth was still observed. Increasing the concentrations beyond $M/7500$ valine, $M/1700$ leucine and $M/2500$ *iso*leucine did not increase growth significantly. These quantities were therefore used in all subsequent tests.

Natural isomers.

So far in all work synthetic preparations of valine, leucine and *iso*leucine were used. Krebs (1935) showed that the action of glutaminase was inhibited by the non-natural isomer ($d(-)$-glutamic acid) of glutamic acid. It was thought possible that the inhibitory effects described above might be due to the non-natural isomer of the synthetic racemic mixtures. The experiments were, therefore, repeated with the natural isomers. $l(+)$-Valine and $l(+)$-leucine gave results identical to those given by their synthetic preparations. $l(+)$-*Iso*leucine, however, not only produced no inhibition in a concentration of $M/2500$ in a medium lacking both valine, and leucine, but actually had a stimulating effect, producing growth overnight little inferior to that in the complete medium. Now, of the three amino-acids, natural *iso*leucine was the only one which was used direct from the manufacturers without previous attempts at purification. Other amino-acids are likely to be associated with it. Chemically, leucine and valine are likely contaminants, in which case the toxicity of *iso*leucine would not only be antagonized, but its stimulating effect in the presence of valine and leucine would be shown. The direct stimulating effect of the preparation could thus be accounted for. That valine and leucine were also present was suggested by the results obtained when either valine or leucine was combined with the natural *iso*leucine ; growth was, if anything, improved, although no growth would have occurred with the synthetic *iso*-leucine unless both together were present. It is likely that the natural *iso*leucine supplied the third amino-acid (either valine or leucine) necessary for growth.

By recrystallizing natural *iso*leucine from aqueous alcohol, my colleague

Dr. H. McIlwain obtained a preparation which was less soluble than the original sample. This did not inhibit growth in concentrations of $M/2500$ and $M/12{,}500$, but completely prevented growth at a concentration of $M/62{,}500$. The " stimulating factor " could thus be diluted out. On still further reducing the concentration, growth reappeared at approximately the limiting concentration of the synthetic preparation. This sample was tested for leucine by combining it with valine and using the mixture for growth tests as outlined above, and *vice versa*. Although growth was obtained with $M/7500$ valine in a concentration of $M/62{,}500$, suggesting that leucine was still present, no growth was obtained with $M/1700$ leucine with $M/12{,}500$ and only poor growth with $M/2500$, showing that little valine could be present.

These results show that the sample of natural *iso*leucine contained impurities (probably valine and leucine). When these were removed by recrystallizing and " diluting-out ", the sample was similar to the synthetic preparation. Another sample obtained from Hoffmann-La Roche did not possess this impurity and behaved like the synthetic preparation.

Late growth in the absence of valine, leucine and iso*leucine.*

In view of the small concentrations of these amino-acids which would effect growth, it was thought that late growth in their absence might be due to traces present as contaminants in other amino-acids. These could only be present in the natural amino-acids (proline, oxyproline, histidine, arginine, tryptophan and cystine) or in glutamine. Although these substances were of high commercial grade and had been subjected to purification processes, impurities could not be excluded with certainty. An investigation was therefore made to find out which of these amino-acids could be left out. As mentioned above, it was necessary to keep cystine, but all the other natural amino-acids and glutamine could be excluded from the medium. Growth on this mixture of synthetic amino-acids (alanine, glycine, aspartic acid, glutamic acid, phenylalanine, tyrosine, methionine, valine, leucine and *iso*leucine) *plus* cystine was nearly as good as on the complete medium, but was delayed about 12 hours, presumably due to the time required for the synthesis by the organism of those amino-acids which had been omitted. The omission of valine, leucine, or *iso*leucine *singly* from this medium gave the same results as on the complete medium. When, however, all three were excluded *together*, instead of the late growth obtained when the natural amino-acids were present, complete absence of growth (6 days) resulted. The quantitative relations between valine, leucine and *iso*leucine were the same as on the complete medium. As before, some growth was obtained with valine and leucine alone ; this was improved by the addition of *iso*leucine. Since no growth occurred in the absence of all three amino-acids, the extremely small quantities which could support growth could be appreciated better than on the complete medium. It was found that $M/23 \times 10^{-6}$ valine, $M/5 \times 10^{-6}$ leucine and $M/7 \times 10^{-6}$ *iso*leucine produced growth in 2 days, and some growth was even produced by about $M/1 \times 10^{-9}$ valine and $M/1 \times 10^{-8}$ leucine and *iso*leucine in 5 days.

These results show that late growth in the absence of valine, leucine and *iso*leucine is due to the presence of one or more of the natural amino-acids.

Experiments were therefore undertaken to find out which amino-acids were concerned. No single natural amino-acid, added to the synthetic mixture (*plus* cystine) in the concentration present in the complete medium, was able to support growth in the absence of valine, leucine and *iso*leucine, but a combination of arginine and histidine was. None of the other natural amino-acids appeared to be involved. It may be assumed, therefore, that arginine and histidine were responsible for the late growth in the complete medium when valine, leucine and *iso*leucine were omitted. Quantitative tests showed that rather larger concentrations than those present in the complete medium were required to produce the best growth. In subsequent tests they were each used in a concentration of $M/2000$ instead of $M/4000$.

As suggested above, the action of arginine and histidine might be due to small quantities of leucine and valine present as impurities. This cannot explain their whole action, as they had an accelerating effect on growth in the presence of adequate concentrations of valine and leucine. If such contamination was present, since each singly was unable to support growth, one would have to contain only leucine and the other only valine. To test this each was combined with very small concentrations of valine or leucine. In no case did growth occur. Finally, Dr. H. McIlwain reprecipitated each with 10 per cent. phosphotungstic acid in 5 per cent. H_2SO_4 (phosphotungstic acid and H_2SO_4 being removed with $Ba(OH)_2$). Growth was still as good as with the original preparations.

These results suggest that the effect of arginine and histidine is not due to the presence of impurities. This can only be finally established by the use of synthetic preparations. It has not been possible to obtain synthetic arginine, but synthetic histidine combined with natural arginine is as effective as a combination of the two natural amino-acids.

Nor*leucine*.

s-*Nor*leucine was similar to s-*iso*leucine in its action, but was less toxic. A concentration of $M/12{,}500$, when added to a medium lacking valine, leucine and *iso*leucine, completely inhibited growth, but with $M/62{,}500$ growth occurred with only a slight delay. Leucine ($M/1700$) and valine ($M/7500$) together antagonized this effect, but each singly was inactive. Unlike *iso*leucine, in the presence of leucine and valine, *nor*leucine did not improve growth.

Serine and Threonine.

In work on the growth of *Streptococcus pyogenes*, Fildes (unpublished) obtained evidence as to the existence of a relationship between serine and threonine similar to that between valine and leucine described above. At Dr. Fildes' suggestion, these amino-acids were tested for their effect on the growth of *B. anthracis*. In the complete medium, neither s-threonine or s-serine in a concentration of $M/500$ had any effect on growth, but serine in a concentration of $M/100$ delayed growth slightly. When valine, leucine and *iso*leucine were removed from the medium, a marked inhibitory effect with both threonine and serine was obtained (Table IV).

Since the toxic effect of threonine and serine was not found when leucine, valine and *iso*leucine were all present, it follows that one or more of these

TABLE IV.—*The Effect of Threonine and Serine on Growth. Basal Medium Lacking Valine, Leucine and* Isoleucine.

		Growth.						
		Hours.				Days.		
		17.	24.	41.	47.	3.	4.	14.
Basal medium alone	. . .	0	0	+	++	++	++	..
Ditto + threonine	*M*/2500 . .	0	0	0	0	0	0	0
,, ,,	*M*/12,500 . .	0	0	0	0	tr.	++	..
,, ,,	*M*/62,500 . .	0	0	0	tr.	+	++	..
,, ,,	*M*/312,500 .	0	0	+	+	++	++	..
,, ,,	*M*/1,562,500 .	0	0	+	++	++	++	..
,, + serine	*M*/2500 . .	0	0	0	0	0	0	0
,, ,,	*M*/12,500 . .	0	0	0	tr.	++	++	..
,, ,,	*M*/62,500 . .	0	0	tr.	+	++	++	..
,, ,,	*M*/312,500 .	0	0	+	++	++	++	..
,, ,,	*M*/1,562,500 .	0	0	+	++	++	++	..

amino-acids was able to antagonize it. Not one of the amino-acids singly was able to " neutralize " the toxic effect of serine ; valine (*M*/7500) and leucine (*M*/1700) together, however, were. The inhibitory effect of threonine was not removed by either leucine or *iso*leucine ; valine, however, in a concentration of *M*/187,500 (which itself would have prevented growth in a medium lacking leucine), not only " neutralized " the toxicity of *M*/2500 threonine, but growth was actually accelerated (trace of growth in 24 hours), *i. e.* valine and threonine are mutually antagonistic. With leucine also present, growth was still further improved.

A mutual relationship was also found to exist between serine and threonine. In a medium without leucine, valine or *iso*leucine, *M*/312,500 threonine removed the toxic effect of *M*/100 serine. The optimum concentrations were *M*/500 serine and *M*/312,500 threonine. Moreover, a combination of these amino-acids in these concentrations had a marked accelerating effect on growth, not only in the medium lacking valine, leucine and *iso*leucine, but also on the full amino-acid medium. Whereas growth normally only reached a maximum in 24 hours, with serine and threonine present it was maximum in 18 hours.

Summarizing : threonine and serine are both toxic singly in a medium lacking valine, leucine and *iso*leucine. The toxicity of threonine is removed by valine or serine, but not by leucine or *iso*leucine. The toxicity of serine is removed by adding threonine, or valine *plus* leucine, but not by each singly. A combination of serine and threonine in suitable concentrations enhances growth in the complete amino-acid medium.

α-Amino-butyric Acid, β-Hydroxybutyric Acid and β-Hydroxyglutamic Acid.

Owing to a certain resemblance in chemical structure between these substances and threonine and serine, they were tested for biological activity in concentrations of *M*/1000 and *M*/5000. Only s-α-amino-butyric acid was active. On the complete medium little effect was found, but on the medium in which leucine, valine and *iso*leucine were omitted, *M*/5000 completely inhibited growth. The limit of toxicity was found to be about *M*/25,000.

$M/7500$ valine " neutralized " the toxicity of $M/5000$ and $M/25{,}000$, but not of $M/1000$, α-amino-butyric acid, *i. e.* α-amino-butyric acid and valine are mutually antagonistic in their action. Quantities less than $M/25{,}000$ failed to grow with $M/7500$ valine, since they were insufficient to " neutralize " the toxic effect of the latter. With leucine ($M/1700$) also present, growth with $M/1000$ was obtained.

Utilization of Ammonia as the Sole Source of Nitrogen.

As in the case of *Bact. typhosum* (Fildes, Gladstone and Knight, 1933) and *Staphylococcus aureus* (Gladstone, 1937), by gradually reducing the number of amino-acids in the medium in serial subculture, *B. anthracis* can be " trained " to utilize ammonia as its sole source of nitrogen. Growth was poor and delayed, but could be continued through an indefinite number of subcultures. The spores from such a culture retain their ammonia-assimilating properties. As in the case of an untrained strain, valine, *iso*leucine and serine added singly inhibited growth in the ammonia medium, but leucine and threonine were no longer toxic. The organism in the process of training had not been in contact with any of these amino-acids.

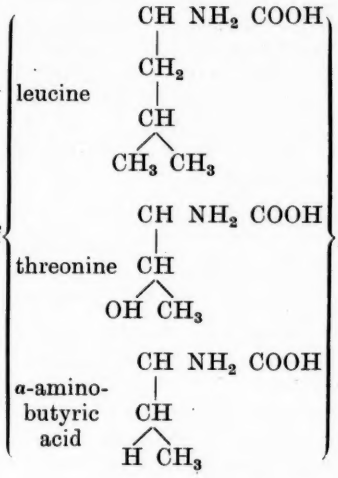
DISCUSSION.

It is well known that amino-acids under certain conditions may be toxic for growth (Gordon and M'Leod, 1926; and others). Recently Nielson and Hartelius (1938) have shown that, in the absence of asparagine or aspartic acid, β-alanine inhibits the growth of *Saccharomyces cerevisiæ*; in the presence of these substances, growth is improved by β-alanine.

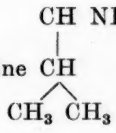
The results recorded in this paper show an inter-relationship between certain amino-acids whereby the toxic effect of one may be counteracted by the addition of one or more of others. Moreover, the amino-acids which are able to " neutralize " the toxic effect are themselves toxic when added singly. We have, therefore, the interesting fact that two substances, both of which are toxic singly, when added together not only allow growth to occur, but in most cases actually improve it. Now the particular amino-acids which appear to be associated in this way have a similarity in chemical structure (Table V). This is suggestive of some common reaction which is necessary for growth, and which can be inhibited by excess of one or other of the amino-acids taking part. The function most easily attributed to amino-acids is the synthesis of bacterial protoplasm. Growth, however, is not dependent on the presence of these amino-acids, but in their absence it is delayed. This does not mean that these amino-acids are not required for growth. When a particular amino-acid accelerates growth (which nevertheless would occur in its absence), all the evidence suggests that such an amino-acid is necessary for growth, but when it is not added it can be synthesized by the organism. It may be assumed, therefore, that all these amino-acids are required for growth, but can be synthesized by the organism. Now it is possible that excess of one may " block " the reaction or enzymes necessary, either for the synthesis of another of similar chemical composition, or for building it when synthesized into bacterial protoplasm. Some strains are less susceptible to the toxic effect than others. For example, the growth of strain 8 is only slightly delayed

TABLE V.—*Similarity in Chemical Composition between Amino-acids Inter-related in their Effect on the Growth of* B. anthracis.

The toxicity of

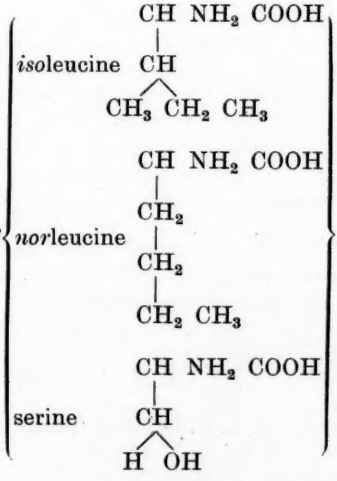


is removed by valine

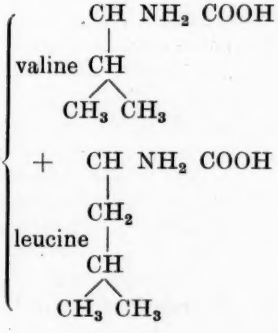


and *vice versa.*

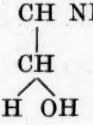
The toxicity of



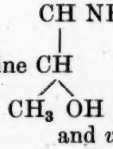
is removed by



The toxicity of serine



is removed by threonine



and *vice versa.*

in the presence of leucine and *iso*leucine and of valine and *iso*leucine. This is probably due to a difference in synthetic ability between strains. Strain 8 is very easily adapted to grow with ammonia as the sole source of nitrogen, *i. e.* to synthesize all the amino-acids it requires from ammonia. If the rate of synthesis of a particular amino-acid is greater than the rate at which the reaction is " blocked ", growth will occur in spite of an excess of the inhibitory amino-acid being present. A similar explanation may be attributed to the fact that the organism may be " trained " to overcome the toxic effect.

Apart from these theoretical considerations, the results may have a certain practical application. In investigations on the amino-acid growth requirements, whether in animal or bacterial nutrition, the procedure adopted has been the obvious one of omitting single amino-acids from a balanced diet and noting the effect on growth. An amino-acid is regarded as " indispensable " if growth is impaired when it is omitted. The results of this paper suggest that, before such a statement should be made, the effect of omitting two or more of these " indispensable " amino-acids together should be examined. It is possible that some may be found to be " indispensable " only in the presence of others.

SUMMARY.

An inter-relationship exists between certain amino-acids of similar chemical composition in their effect on the growth of *B. anthracis*. Valine, leucine or *iso*leucine, added singly to a mixture of amino-acids able to support growth without them, completely prevented growth. When added all together, growth was improved and accelerated. The toxic effect of valine could be counteracted by leucine and *vice versa* ; but that of *iso*leucine could only be " neutralized " by the presence of both valine and leucine. *Nor*leucine was similar to *iso*leucine in its action. Similar inter-relationships were found between valine and threonine, valine and α-amino-butyric acid, and threonine and serine. The toxic effect of serine could also be neutralized by a combination of valine and leucine, but not by each singly. Definite quantitative relationships were found, but these were not necessarily equimolecular.

My thanks are due to Dr. Paul Fildes, under whose direction this work was done, for helpful advice and criticism, and to my colleague Dr. H. McIlwain, who was responsible for the chemical preparations mentioned in the text.

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SURVEY OF PAPERS.

In the second part of their study of the antigen from *Br. melitensis* MILES and PIRIE describe the serological behaviour of the antigen in its native state and after step-wise degradation of various agents. The relationship between the physical properties of the antigen solution and the character of the serum precipitate is discussed (p. 109).

ARGYLL CAMPBELL finds that dust from tarred roads and chimney soot contain carcinogenic agents. The former definitely increases the incidence of primary lung tumours in mice; the latter produces little change. The road dust contains other agents (inorganic substances) which aid the tar in producing its effects. The carbon of the soot mitigates the effects of the tar in the chimney soot (p. 122).

POPE has re-examined the possibility of purifying antitoxic sera by digestion of inactive protein with proteolytic enzymes (mainly pepsin). Working with diphtheria antitoxic serum he has determined the conditions necessary for maximum digestion of inactive protein and minimum loss of antitoxin activity. Samples of plasma from different horses show considerable variation in response to pepsin action; usually plasma from horses which have responded well to immunization give best results.

It is concluded that peptic digestion alone does not provide a suitable method for antitoxin purification, since the degree of purification is rather low (up to 4 times), and the products of the breakdown of inactive protein difficult to separate from the antitoxin (p. 132).

No effective method has yet been devised of inducing a satisfactory immunity against Aujeszky's virus disease of animals (pseudo-rabies). GLOVER has endeavoured to adapt the virus to the developing egg in the hope that the infective agent would undergo mutation and serve as an immunizing agent.

The virus has been successfully propagated on the egg. Some evidence of mutation has been obtained, but the virus is not yet sufficiently attenuated to permit its employment for purposes of vaccination (p. 150).

DOWNIE's finding that histological differences occurred in the lesions produced by the viruses of cowpox and vaccinia led him to investigate these two viruses by serological methods.

He now shows that rabbits immunized with either virus are immune to both, and that immune sera prepared with either virus neutralize both. However, cross-absorption experiments with hyper-immune sera and elementary body suspensions show that while the homologous virus removes the antibodies for both viruses, the heterologous virus absorbs chiefly its own antibodies. These results indicate that the viruses of cowpox and vaccinia are closely related, but not identical, as previously assumed by many workers (p. 158).

FULLER and MAXTED note that previous observations on peroxide formation by hæmolytic streptococci have been without reference to the group or type of the strain. They show that all known types of group A hæmolytic streptococcus form peroxide with the exception of type 3.

It is remarkable that these latter strains which produce no peroxide in aërobic culture are inhibited by catalase in the medium (p. 177).

MCNAIR SCOTT and ELFORD have sought to determine the particle size of the infective agent of lymphocytic choriomeningitis as it exists in broth extracts of infected mouse brain. The evidence furnished by the two methods applied, namely ultrafiltration and centrifugation analysis, has indicated the probable diameter of the virus to be 40–50mμ. (p. 182).

GLADSTONE grows *B. anthracis* in a chemically defined amino-acid medium. On omitting certain amino-acids singly growth ceases, and the amino-acid would thus appear to be indispensable. However, if in addition another particular amino-acid is omitted, growth occurs, showing that the first amino-acid was not indispensable. It would appear that each amino-acid is really inhibitory, but stimulating when both are present. These observations may have a bearing on conclusions drawn from nutritional studies in animals (p. 189).

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